



WASHINGTON STATE PATROL

CASEWORK

STR ANALYSIS PROCEDURES

CRIME LABORATORY DIVISION

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INTRODUCTION

It is important to note that procedure development is an ongoing process. The DNA Technical Leader should be consulted for significant changes to any of the following procedures or for developing additional procedures. These changes shall be discussed at a functional area meeting (or through e-mail or phone conference calls) prior to being incorporated into this manual. Allowances (non-significant changes) can be made for adapting procedures (e.g. volume, tube size/type, spin times, incubation times) to accommodate unusual case samples and situations. Allowances to adapt a standard procedure are a recognized part of casework, as these may be necessary to meet the requirements of certain cases or samples. These deviations must be scientifically sound and should be documented in the casework notes.

The standard method of analysis of DNA conducted by the Washington State Patrol Crime Laboratory (WSPCL) for criminal cases and for the convicted offender database is by the analysis of Short Tandem Repeat (STR) regions by Polymerase Chain Reaction (PCR). The WSPCL examines 27 loci: the original Combined DNA Index System (CODIS) core 13 loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, vWA, TPOX, D18S51, D5S818, and FGA); the additional newly adopted 7 CODIS core loci (D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045); Amelogenin and DYS391 (for sex discrimination); Penta D, Penta E and SE33 for increased discrimination; and rapidly mutating YSTR loci DYS570 and DYS576.

PCR is a method used for the amplification of a specific DNA segment whereby two oligonucleotides (primers) anneal to opposite strands and flank a DNA region to be copied. The synthesis reaction is repeated for a number of cycles and results in the exponential accumulation of the specified DNA segment, the termini of which are defined by the 5' ends of the primers used in the reaction.

EXTRACTION

DNA suitable for PCR-based typing is obtained by either an automated approach like those used by the QIAGEN BioRobots or a manual approach like the organic solvent extraction method. The organic method can be done in combination with other purification methods such as EZ1 DNA purification.

A differential lysis procedure is used to separate sperm cell DNA from other cellular sources of DNA, such as vaginal epithelial cell DNA. To accomplish this, advantage is taken of a particular characteristic of sperm cell membranes: sperm cells are resistant to lysis unless a reducing agent, such as dithiothreitol (DTT), is present. Therefore, other cells in the presence of sperm cells can be preferentially lysed with Proteinase K, the mixture spun, and the supernatant containing the non-sperm cell DNA removed. The pelleted sperm cells can then be lysed using Proteinase K and DTT. It should be noted that the differential lysis procedure is frequently not 100% effective in separating sperm cell DNA from other cellular sources of DNA. The analyst has the discretion to choose a procedure that is appropriate for a particular sample.

HUMAN DNA QUANTIFICATION

Plexor® HY System

The quantitative PCR (qPCR) kits provide reagents and protocols necessary for the rapid and sensitive quantification of human nuclear DNA. The role of the Plexor® HY System is simultaneous detection and quantification of autosomal and Y-chromosomal DNA. Promega's Plexor® HY System autosomal primers amplify a 99bp sequence from the RNU2 locus located on chromosome 17. The Y-chromosome primers target a 133bp sequence from the testis-specific protein, Y-encoded (TSPY) locus within the DYZ5 region of the Y chromosome. The procedure is based on the quenching of a fluorescent reporter due to the site-specific incorporation of dabcyI-iso-dGTP (a modified nucleotide in the dNTP mix that binds opposite iso-C, a modified nucleotide on one of the PCR primers at the 5'-labeled end). During elongation, dGTP is incorporated opposite iso-C; the proximity of dabcyI to the reporter (fluorescein) quenches the fluorescence thus decreasing the fluorescent signal as the PCR amplified product accumulates. Also included in each of the kits is an internal PCR control (IPC) that can detect the presence of PCR inhibitors. This is a non-naturally occurring sequence. Additionally, a passive reference dye has been included within Promega's Plexor HY System to reduce the impact of instrument-specific signal fluctuation. The qPCR is performed in the Applied Biosystems 7500 Real-Time PCR System instrument.

PowerQuant™ System

The PowerQuant™ System is a five-dye, four-target hydrolysis probe-based qPCR multiplex that amplifies multicopy targets to quantify the total human and human male DNA present in a sample. The human autosomal primers amplify an 84-base pair sequence while the Y chromosome primers amplify two multicopy loci (81bp and 136bp sequences). The use of two multicopy loci minimizes the effect that variation in copy number of any given marker can have on [Auto]/[Y] ratios and increases the sensitivity for male DNA. The system also detects degradation by targeting a longer amplicon (294bp) derived from a different region of the same locus as the autosomal target. Due to its greater length, the degradation amplicon is more susceptible to degradation and the presence of inhibitors. The ratio of DNA concentrations determined with the autosomal and degradation targets ([Auto]/[D] ratio) can be used to evaluate the degree of degradation. Additionally, the PowerQuant™ System includes an internal PCR control (IPC) to detect inhibitors in an amplification reaction. The IPC primers produce an amplified product that is 435bp and is the longest target in the PowerQuant™ System. This makes it more susceptible to inhibitors than the other targets in the multiplex.

Data generated using the PowerQuant™ System can help determine whether an unknown DNA sample is suitable for short tandem repeat (STR) analysis, what is the appropriate STR system to use (e.g., autosomal or Y-STR) and whether the DNA is degraded or PCR inhibitors are present. This information may be used to guide decisions regarding sample processing including resampling, optimizing template volume to add to an STR amplification, adjusting for degradation or inhibition in samples, or halting analysis.

A standard curve is required to determine the DNA concentration of unknown DNA samples. This standard curve is generated using the amplification results from a dilution series of a male DNA standard of known concentration.

AMPLIFICATION

STR PCR amplification is performed using the ABI GeneAmp® PCR System 9700 thermal cycler instrument.

The polymerase used requires heat activation. This has the advantage that the enzyme is not active during the setup of the samples. The formation of "primer dimers" is avoided and there is no time constraint placed upon the setup procedure. To activate the enzyme, the amplification cycle includes a hot start enzyme activation at the beginning of the amplification process.

Three steps are involved in each amplification cycle. First, the template DNA is made single-stranded by heat denaturation. In the second step, the temperature is lowered so that annealing of the oligonucleotide primers to the template may occur. This interaction is favored because, initially, the ratio of primer to template is large. Annealing of the PCR primers to the template DNA is responsible for the technique's high level of specificity. Under optimized conditions, annealing of complimentary DNA strands (primers) should only occur at those sites on the template flanking the region to be amplified. In this way, a sequence of several hundred bases can be selected from a background of, in the case of human DNA, 3×10^9 base pairs.

In the third step, the temperature is raised to a point that favors both specific annealing of the primers and extension by the DNA polymerase. These three steps are repeated over and over again to achieve a sufficient quantity of amplified product. The target DNA is amplified at an exponential rate if one assumes a 100% efficient PCR reaction. Even when carefully controlled, a PCR reaction is rarely, if ever, 100% efficient.

Some amplification polymerases add an extra nucleotide (usually adenosine) to the end of a strand of DNA. If this reaction only occurs partially, then DNA of two lengths, differing by one base pair, will be produced from the same template and primers, causing the appearance of a split peak. Having amplified product of uniform size is important in STR analysis, as it is the length of the final product that is detected. A length variance of one base pair is detectable by the system, and therefore undesirable. To avoid this, the addition can either be prevented or conditions chosen so virtually all the amplification product is converted. Since the latter is easier, a 60°C soak is included at the end of polymerization cycle to drive the addition reaction to completion.

DETECTION

STR TYPING KITS

Kits that contain the necessary components to amplify and detect Amelogenin and the 20 core STR loci used by CODIS are commercially available. It is possible to detect and distinguish the products of several loci amplified together. This is referred to as multi-component analysis. The Fusion 6C kit allows for co-amplification and fluorescent detection of the 20 CODIS core loci as well as Amelogenin and DYS391 for sex determination and Penta D, Penta E, SE33, DYS570, and DYS576 for higher discrimination.

Fusion 6C kit loci

LOCUS	SIZE RANGE (bp) ¹	Dye Color ²
Amelogenin	80 – 89	Blue
D3S1358	90 – 151	Blue
D1S1656	152 – 209.50	Blue
D2S441	211 – 252	Blue
D10S1248	254 – 302.50	Blue
D13S317	304.50 – 357	Blue
Penta E	362 – 482	Blue
D16S539	74 – 129.4	Green
D18S51	131 – 217.5	Green
D2S1338	221.5 – 304	Green
CSF1PO	313 – 366.5	Green
Penta D	373.5 – 470	Green
TH01	65 – 118	Yellow
vWA	121 – 192	Yellow
D21S11	197 – 266.5	Yellow

D7S820	268 – 315.5	Yellow
D5S818	317.50 – 380	Yellow
TPOX	390 – 448	Yellow
D8S1179	66 – 129.8	Red
D12S391	130.10 – 190.5	Red
D19S433	192 – 255	Red
SE33	270 – 429	Red
D22S1045	430 – 478	Red
DYS391	79.5 – 131	Purple
FGA	134 – 299	Purple
DYS576	302 – 370	Purple
DYS570	380 – 464	Purple

¹Values obtained from the GMID-X panels; the estimated size generated by the CE unit will be different.

²The dyes used are FL-6C (blue), JOE-6C (green), TMR-6C (yellow), CXR-6C (red), TOM-6C (purple), and WEN-6C (orange). For more information on the loci and dyes, see the Fusion 6C user's manual.

As a supplemental DNA analysis method, the WSPCL also employs the AmpF Φ STR[®] Yfiler[®] and PowerPlex[®] Y23 System amplification kit for the detection of male haplotypes. The AmpF Φ STR[®] Yfiler[®] PCR Amplification Kit is a Short Tandem Repeat (STR) multiplex that amplifies 17 Y-STR loci. These 17 loci are found on the non-recombining region of the Y chromosome, allowing the amplification of only human male DNA. The alleles at each locus are inherited as one linked block of genetic information that is passed down through a paternal lineage. This technology allows for the analysis of forensic casework samples that contain small amounts of male DNA in the presence of large amounts of female DNA.

Yfiler[®] kit loci

LOCUS	SIZE RANGE (b.p.) ²	DYE COLOR
DYS456	100 – 127	Blue – 6-FAM [™]
DYS389I	134 – 178	Blue – 6-FAM [™]
DYS390	185 – 245	Blue – 6-FAM [™]
DYS389II	246 – 302	Blue – 6-FAM [™]
DYS458	133 – 165	Green - VIC [®]
DYS19	167 – 219	Green - VIC [®]
DYS385 a/b ¹	235 – 323	Green - VIC [®]
DYS393	104 – 144	Yellow - NED [™]
DYS391	146 – 181	Yellow - NED [™]
DYS439	192 – 236	Yellow - NED [™]
DYS635	241 – 274	Yellow - NED [™]
DYS392	286 – 335	Yellow - NED [™]
Y GATA H4	114 – 150	Red - PET [®]
DYS437	174 – 210	Red - PET [®]
DYS438	215.5 – 256.5	Red - PET [®]
DYS448	273 - 332	Red - PET [®]

¹ DYS385 a/b is a duplicated locus on the Y chromosome, and is counted as 2 loci when describing.

² Size in actual base pairs; the estimated size generated by the CE unit will be different.

The PowerPlex[®] Y23 System PCR Amplification Kit is a Short Tandem Repeat (STR) multiplex that amplifies 23 Y-STR loci. These 23 loci are found on the non-recombining region of the Y chromosome,

allowing the amplification of only human male DNA. The alleles at each locus are inherited as one linked block of genetic information that is passed down through a paternal lineage. This technology allows for the analysis of forensic casework samples that contain small amounts of male DNA in the presence of large amounts of female DNA. Both DY570 and DY576 are classified as rapidly mutating loci.

Y23 STR Locus	Size Range of Allelic Ladder Components (b.p.) ²	Dye/Color
DYS576	97-145	Fluorescein/Blue
DYS389 I	147-179	Fluorescein/Blue
DYS448	196-256	Fluorescein/Blue
DYS389 II	259-303	Fluorescein/Blue
DYS19	312-352	Fluorescein/Blue
DYS391	86-130	JOE/Green
DYS481	139-184	JOE/Green
DYS549	198-238	JOE/Green
DYS533	245-285	JOE/Green
DYS438	293-343	JOE/Green
DYS437	344-380	JOE/Green
DYS570	90-150	TMR-ET/Yellow
DYS635	150-202	TMR-ET/Yellow
DYS390	207-255	TMR-ET/Yellow
DYS439	263-307	TMR-ET/Yellow
DYS392	314-362	TMR-ET/Yellow
DYS643	368-423	TMR-ET/Yellow
DYS393	101-145	CXR-ET/Red
DYS458	159-215	CXR-ET/Red
DYS385 a/b ¹	223-307	CXR-ET/Red
DYS456	316-364	CXR-ET/Red
Y-GATA-H4	374-404	CXR-ET/Red

¹ DYS385 a/b is a duplicated locus on the Y chromosome, and is counted as 2 loci when describing.

² Size in actual base pairs; the estimated size generated by the CE unit will be different

MULTICOMPONENT ANALYSIS

Multicomponent analysis uses two separate properties to distinguish between loci: length variance and fluorescent labels.

LENGTH VARIANCE

The amplified product from each locus will vary in length, dependent upon the number of repeats found in a particular STR polymorphism. By careful selection of primers, it is possible to produce amplified products for several loci, all of which fall into discrete and separate size ranges. Thus a particular locus can be identified by the size of its alleles.

FLUORESCENT LABEL

Different fluorescent labels can be attached to the primers used in the amplification process. The emission spectra of these labels are sufficiently different such that the contribution made by each label in a mixture can be calculated both quantitatively and qualitatively. Thus even if two separate DNA fragments co-migrate they can be differentiated by their fluorescent labels.

By careful selection of both primers and fluorescent dyes, several different co-amplified loci can be distinguished based both upon their fluorescence and the size of the fragment lengths. Theoretically, it is possible to detect a large number of co-amplified loci by a combination of these two detection methods.

Each of the dyes employed detects loci in discreet size ranges. This allows for the detection of multiple loci at one time. Each amplification kit dedicates one dye to label the internal size standard.

GENETIC ANALYZER (CAPILLARY ELECTROPHORESIS (CE) UNIT)

The genetic analyzer (CE unit) separates DNA fragments based upon their size and also upon their attached fluorescent labels using capillary electrophoresis. A window in the capillary is continually being illuminated by a laser. As fragments of DNA with fluorescent labels pass by the window, they are excited and the resulting emitted light collected and analyzed.

The Applied Biosystems 3500 Genetic Analyzers is currently being used by the WSPCL for the analysis of DNA fragments with fluorescent labels.

INTERNAL SIZE STANDARDS

An appropriate internal size standard is added to each sample prior to its electrophoresis. The internal size standard consists of several amplified fragments of DNA, all of which have been sequenced and are of known length. The migration time of each fragment is recorded and these data are used to calculate an estimated length for the other DNA fragments that are detected.

INTERPRETATION OF RESULTS

The data generated is analyzed using software programs available from Applied Biosystems.

GeneMapper® ID-X program

The GeneMapper® ID-X program converts the raw data from the AB CE instruments into discreet peaks, assigns a size to those peaks based upon their estimated length in base pairs, converts the estimated fragment sizes in base pairs to designated alleles by comparing the fragment sizes to the known alleles in a standard called an allelic ladder. The program also displays that data in the form of an electropherogram.

NON-DIFFERENTIAL LYSIS PROCEDURE

1. Place sample (e.g. cutting from bloodstain - up to 1 centimeter²) into a 2 ml COSTAR® Spin-X® tube basket assembly (or other tube basket assembly that has been performance tested and found to be suitable).
2. Add 500 µl TNE, 25 µl 20% Sarkosyl, and 7.5 µl 20mg/ml proteinase K to each tube. Vortex briefly.
3. Incubate at 56°C for a minimum of 3 hours or overnight (24-hour maximum).
4. Pulse spin. Transfer cutting into a Spin-X® basket insert and place the basket insert into the tube containing the stain extract. Spin at maximum speed in a microcentrifuge for 3 to 5 minutes. If the LySep basket is used for lysis, the pulse spin and substrate transfer steps are not necessary. The LySep basket and tube can be placed directly into a microcentrifuge and spun at maximum speed for 3 to 5 minutes.
5. Remove and discard basket insert and cutting.
6. The digested samples are now ready for extraction using one of the following procedures:
 - a. Organic Extraction Procedure – Phenol/chloroform/isoamyl alcohol followed by concentration/purification by a concentration unit (Amicon, Microcon, etc.).
 - b. QIAGEN EZ1 Biorobot® workstation

DIFFERENTIAL LYSIS PROCEDURE FOR SEMEN STAINS

1. Using a clean surface for each sample processed, place the sample in a 2.0 ml COSTAR® Spin-X® tube basket assembly (or other tube basket assembly that has been performance tested and found to be suitable).
2. If doing the initial evaluation, add 100-1000 µl of sterile PBS to each of the 2.0 ml tubes and incubate at 37°C for a minimum of 30 minutes. Otherwise, proceed to step 7.
3. Transfer the substrate material into a Spin-X® basket insert. Place the basket insert into the tube containing the stain extract and centrifuge for 5-7 minutes.
4. Remove the basket insert with the substrate material from the extract tube and set aside. (Save for possible later use below.)
5. While being very careful not to disturb the cell pellet in the Spin-X® tube, remove most of the supernatant fluid (~ 65 µl if extracted in 100 µl) from the extract and transfer into a separate 1.5 ml labeled tube. This will be used for conventional serological tests as necessary. Freeze supernatant until use.
6. Re-suspend the cell pellet in the remaining fluid in the Spin-X® tube and place ~3 µl of the suspended material on a microscope slide, heat fix, and then Christmas tree stain. Examine microscopically for sperm and epithelial cell quantity. Depending on the microscopic results, proceed as follows:
 - a. If the extraction yields a sufficient number of sperm, proceed to step 7. However, if a neat semen source is suspected due to the case scenario, the analyst may choose to skip to step 14.
 - b. If no sperm or an insufficient number of sperm are observed and sample permits, additional sample may be extracted. If no sperm are observed, a p30 analysis on the aqueous supernatant (from Step 5) may be warranted.

NOTE: The analyst can choose to delay the micro exam for sperm as described in step 6 of the STR Casework Procedures Manual until after the sperm wash in step 12 or 13 is completed. This can only be done during the examination of oral swabs and genital/anal swabs collected for sexual assaults. A second exam after step 13 is optional.

7. To the remaining cell pellet suspension (or original stain), add ~ 50 µl of sterile dH₂O, 400 µl TNE, 25 µl 20% Sarkosyl, and 2.5 µl of 20 mg/ml proteinase K. For increased yield, the substrate material (previously placed in the basket insert) from step 4 above may be added back to the tube. Vortex briefly.
8. Incubate at 37°C for 1 to 2 hours. (Sometimes, it may be appropriate to lyse for a shorter time.)
9. Centrifuge for 5-7 minutes. (If the substrate was added back to the tube, pulse spin and transfer the substrate into a Spin-X® basket insert and place the basket into the tube before centrifuging. If a Spin-X® basket insert was used, remove the basket and discard the substrate.)

10. While being careful not to disturb the cell pellet, remove the supernatant fluid from the extract and place it into a new, labeled 1.5 ml tube. This supernatant is the non-sperm fraction. Analysis of the non-sperm fraction resumes at step 16. The pellet remaining in the tube is the sperm cell pellet.
11. Wash the sperm cell pellet by re-suspending it in 500 μ l sperm wash buffer and vortex briefly. (The sperm cell fraction tubes can be placed into a floating rack and sonicated for 15 minutes, if desired.)
12. Centrifuge the sperm cell fraction tubes for 5-7 minutes. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet.
13. Routinely, wash two more times with 500 μ L sperm wash buffer, for a total of three washes of the cell pellet (see exceptions below). Remove supernatant after each wash.

NOTE: If a large quantity of epithelial cells relative to sufficient sperm cells was observed during the microscopic exam, additional washes may be beneficial. However, if the quantity of sperm cells observed during the microscopic exam was low, a minimum of one wash may be sufficient, and the washes advised in step 13 may not be warranted due to loss of sperm.

NOTE: If the quantity of sperm cells observed during the microscopic exam was low, a second microscopic examination is recommended at this time. To aid in microscopic staining and subsequent observation, PBS can be substituted for the last wash in the series. Re-suspend the pellet in the remaining fluid and prepare a microscope slide as previously described.

14. To the tube containing the washed pellet, add 150 μ l TNE, 50 μ l 20% Sarkosyl, 15.6 μ l 1M DTT, 150 μ l sterile dH₂O and 5 μ l of 20 mg/ml proteinase K. Vortex briefly.
15. Incubate at 37°C for 2 hours. The sample may remain overnight at 37°C.
16. The digested samples are now ready for extraction using one of the following procedures:
 - a. Organic Extraction Procedure – Phenol/chloroform/isoamyl alcohol followed by concentration/purification by a concentration unit (Amicon, Microcon, etc.).
 - b. QIAGEN EZ1 Biorobot® workstation.

LYSIS PROCEDURE FOR HAIR

1. Cut off up to 10 mm of the root end and place in a 1.5 ml microcentrifuge tube.
2. If needed, a substrate sample can be obtained by cutting off an adjacent 5 to 10 mm of shaft and placing into a separate 1.5 ml microcentrifuge tube.

NOTE: Hair may contain cellular material on the surface that may or may not originate from the donor.

3. Add to each tube 500 µl Digest buffer*, 7.5 µl 20 mg/ml proteinase K and 20 µl 1 M DTT. Vortex briefly. Make sure hair is completely immersed.
4. Incubate at 56°C for a minimum of 6 hours or overnight (24-hour maximum).
5. Pulse spin in microcentrifuge to remove condensate from lid.
6. The digested samples are now ready for extraction using one of the following procedures:
 - a. Organic Extraction Procedure – Phenol/chloroform/isoamyl alcohol followed by concentration/purification by a concentration unit (Amicon, Microcon, etc.).
 - b. QIAGEN EZ1 Biorobot® workstation.

*Note that Digest buffer is made up of the same components at the same concentrations as Sperm Wash Buffer. Sperm Wash Buffer will substitute.

RECOVERING SLIDE-MOUNTED HAIRS OR SEMEN SMEARS

A scribe can be used to score the coverslip around the hair root portion to be removed, and either process below can be used to remove the scored section of coverslip:

1. The coverslip may be removed by carefully pipetting a suitable solvent (i.e., xylene or toluene*) around the edges of the coverslip. If the coverslip does not loosen and come off, the entire slide can be placed in a petri dish and covered with solvent for one or more hours until the coverslip has loosened. Beware of multiple hairs on one slide, however.
2. The coverslip may also be removed by freezing the slide in a -20°C freezer for at least 20 minutes, then prying the coverslip off with a scalpel.

Pick out the hair (or hair root or scrape the semen smear) and place in a 1.5 ml microcentrifuge tube. Wash in 500µl xylene or toluene to remove excess Permunt. Follow this with a wash in 500µl ethanol followed by a final rinse in sterile dH₂O.

Proceed with the appropriate extraction procedure.

*NOTE: Solvents such as xylene and toluene are carcinogenic. Wear gloves and work in a fume hood. Take working with low level DNA precautions.

QIAGEN EZ1® PRETREATMENT PROTOCOLS

When diluted Buffer G2 is used, dilute Buffer G2 in sdH₂O using a ratio of 1:1.

FORENSIC SAMPLES

1. Place the forensic sample (e.g. cutting from bloodstain into a tube basket assembly).
2. Add a sufficient volume (a minimum of 190µl to a maximum of 530µl) of neat G2 or diluted G2 to cover the sample (if the sample has absorbed some or all of the buffer, additional volume may be added.)
3. Add 10 µl 20 mg/ml proteinase K and mix thoroughly by vortexing.
4. Incubate at 56°C for 15 minutes up to overnight. Longer incubation times can be used for low level DNA content samples while the shorter incubation times can be used for higher level DNA content samples.
5. Pulse spin. Transfer cutting into a basket insert and then place the basket insert into the tube containing the stain extract. Spin at maximum speed in a microcentrifuge for 5 minutes. Remove and discard basket insert and cutting. Note: If the LySep basket is used for lysis, the pulse spin and substrate transfer steps are not necessary. The LySep basket and tube can be placed directly into a microcentrifuge and spun at maximum speed for 3 to 5 minutes. Certain samples may not require the use of a spin basket. If a spin basket is not used, proceed to step 6.
6. If volume is ~200µl, continue with EZ1 DNA Purification: Trace Protocol or Tip Dance Protocol (if substrate is appropriate). If volume is >200µl, continue with EZ1 DNA Purification: Large Volume Protocol.

HAIR SAMPLES

1. Place the hair sample in a 2ml sample tube (provided in QIAGEN kit).
2. Add 180µl neat Buffer G2 to the sample.
3. Add 10µl 20mg/ml proteinase K and 10µl 1 DTT solution. Mix thoroughly by vortexing for 10 seconds.
4. Incubate at 56°C for at least 6 hours. Pulse spin.
5. Add another 10µl 20mg/ml proteinase K and 10µl 1M DTT solution. Mix thoroughly by vortexing for 10 seconds.
6. Incubate at 56°C for at least 2 hours until the hair sample is completely dissolved. Pulse spin.
7. Continue with EZ1 DNA Purification: Trace Protocol or Tip Dance Protocol.

ORGANIC EXTRACTION PROCEDURE – PHENOL/CHLOROFORM/ISOAMYL ALCOHOL

This procedure is for the isolation of DNA following the lysis/digestion of forensic samples. Wear appropriate protective gear. Perform the following extraction in an appropriate fume hood.

1. Add 500 μ l phenol/chloroform/isoamyl alcohol to the digested stain sample. Vortex until a milky emulsion forms. Spin in a microcentrifuge for 3 to 5 minutes. The DNA will be in the upper aqueous phase.
2. Transfer the upper aqueous layer into a clean 1.5 ml microcentrifuge tube, being careful not to disturb the interface. (If necessary, repeat steps 1 and 2 until the interface is clean.)
 - a. Store refrigerated for a brief time or freeze for long-term storage. Prior to use, vortex briefly and pulse spin.
 - b. Alternatively, the aqueous layer may be transferred directly into a concentration unit.
3. Purify and concentrate the sample(s) using one of the following procedures: Amicon® or Microcon®.

Samples are ready for human DNA quantitation. Use samples immediately for analysis; otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

QIAGEN BIOROBOT EZ1 WORKSTATION

The Qiagen BioRobot® EZ1 and EZ1 Advanced workstations use technology based on DNA binding to magnetic beads followed by washing steps and elution for DNA extraction from forensic casework samples. This process takes the place of the organic phenol/chloroform/isoamyl alcohol extraction and subsequent purification (e.g. Microcon®) steps in forensic DNA analysis.

The sample undergoes lysis/digestion or pre-treatment to prepare it for DNA extraction on the Qiagen BioRobot® EZ1 workstation.

The DNA Investigator Card for the EZ1 workstation has 3 validated DNA purification procedures to select from depending on the desired application: the Trace Protocol, the Large Volume Protocol, and the Tip Dance Protocol.

The DNA purification protocols are designed for isolation of total DNA from forensic samples. The Trace Protocol allows for automated processing of starting volumes up to 200µl while the Large Volume Protocol accommodates starting volumes up to 540µl. The Trace Protocol can also be used to clean up organically extracted genomic DNA preparations that may have co-purified PCR inhibitors present. The Tip Dance Protocol is basically the Trace Protocol with the added benefit of being able to process solid materials (non-fluffy matrix samples such as hair roots and reference blood cards where the danger of the barrier pipette tip clogging is minimal) directly in the sample tube.

EXTRACTION BATCH

A batch should generally not exceed 28 samples. When planning an extraction batch, ensure there are sufficient Reagent Blank samples included in order to adequately cover equal or better sensitivity for the amplifications and analysis to be performed on the extracted DNA. A batch may be processed using two BioRobot EZ1 workstations running at the same time or performing extraction back to back on the same workstation.

EZ1 DNA PURIFICATION: TRACE PROTOCOL

This protocol is designed for isolation of total DNA from forensic samples and enables fully automated processing of starting volumes up to 200µl. It can also be used to clean up organically extracted genomic DNA preparations that may have co-purified PCD inhibitors present. If the starting volume is greater than 200µl, the Large Volume Protocol will have to be used.

Select the appropriate elution volume for the sample being processed.

1. Perform the appropriate lysis procedure (WSP lysis or EZ1 pre-treatment). Label the appropriate number of tubes.
 - a. If performing organic DNA extract clean up, add 1:1 dilute Buffer G2 to reach starting volumes of 195µl.

*NOTE: the addition of 1µl carrier RNA concentration @ 1µg/µl) is not required for known reference samples.

3. Make sure the DNA Investigator protocol card is fully inserted in the EZ1 and turn the instrument on.
4. Press “Start” to display the “Protocols” menu. Select “Trace Protocol” and follow the screen prompts to select the appropriate conditions for the samples being processed and to set up the worktable. Press “Start” to begin the purification procedure.
5. When the protocol ends, “Protocol finished” will be displayed. Retrieve and re-cap the elution tubes.
6. Remove and discard the disposable labware. Follow the cleaning procedure as outlined in the QIAGEN BioRobot EZ1® Workstation Maintenance procedure in this manual. A 20 minute (minimum recommended time) UV decontamination step may be selected following the cleaning procedure.

Note: Due to the chaotropic salts present in the cartridges, do not dispose of them in any bleach or container which may contain bleach as cyanide gas may result.

Samples are ready for human DNA quantitation. Use samples immediately for analysis, otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

NOTE: Prior to quantification, inspect the tubes containing the eluate for the presence of magnetic particles. If magnetic particles are observed, the tube should be applied to a magnetic separator, and the eluate transferred to a clean tube in order to minimize the risk of magnetic particle carryover which may affect the quantification reading.

NOTE: If DNA extracts are likely to be combined with other DNA extracts and/or concentrated using the vacufuge, the samples may be eluted in water, rather than TE. Caution should be used in storing DNA eluted with water since there is no buffering capacity. Extracts in water shall be used the same day or stored frozen until use. If extracts are to be stored at room or refrigerator temperatures, TE or DNASTable must be incorporated.

EZ1 DNA PURIFICATION: LARGE VOLUME PROTOCOL

This protocol is designed for isolation of total DNA from forensic samples and enables fully automated processing of starting volumes up to 540µl.

1. Perform the appropriate lysis procedure (WSP lysis or EZ1 pre-treatment). Label the appropriate number of tubes.
2. Prepare a master mix of Buffer MTL and carrier RNA
 - a. Per sample:
400µl Buffer MTL
1µl carrier RNA @ 1µg/µl
 - b. Mix well and add 400µl of master mix to each labeled screw cap sample tube.
3. If Spin-X tubes were used, transfer the sample lysates from the Spin-X tubes to the 2ml screw-cap sample tubes.
4. Make sure the DNA Investigator protocol card is fully inserted in the EZ1 and turn the instrument on.
5. Press “Start” to display the “Protocols” menu. Select “Large Volume Protocol” and follow the screen prompts to select the appropriate conditions for the samples being processed and to set up the worktable. Press “Start” to begin the purification procedure.
6. When the protocol ends, “Protocol finished” will be displayed. Retrieve and re-cap the elution tubes.
7. Remove and discard the disposable labware. Follow the cleaning procedure as outlined in the QIAGEN BioRobot EZ1® Workstation Maintenance procedure in this manual. A 20 minute (minimum recommended time) UV decontamination step may be selected following the cleaning procedure.

Note: Due to the chaotropic salts present in the cartridges, do not dispose of them in any bleach or container which may contain bleach as cyanide gas may result.

Samples are ready for human DNA quantitation. Use samples immediately for analysis, otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

NOTE: Prior to quantification, inspect the tubes containing the eluate for the presence of magnetic particles. If magnetic particles are observed, the tube should be applied to a magnetic separator, and the eluate transferred to a clean tube in order to minimize the risk of magnetic particle carryover which may affect the quantification reading.

NOTE: If DNA extracts are likely to be combined with other DNA extracts and/or concentrated using the vacufuge, the samples may be eluted in water, rather than TE. Caution should be used in storing DNA eluted with water since there is no buffering capacity. Extracts in water shall be used the same day or stored frozen until use. If extracts are to be stored at room or refrigerator temperatures, TE or DNASTable must be incorporated.

EZ1 DNA PURIFICATION: TIP DANCE PROTOCOL

This protocol is designed for isolation of total DNA from forensic samples and enables fully automated processing of starting volumes up to 200µl with the solid material (hairs, reference blood card, etc.) still in the sample tube.

1. Perform the appropriate lysis procedure (WSP lysis or EZ1 pre-treatment). Label the appropriate number of tubes.
2. If Spin-X tubes were used, transfer the sample lysates from the Spin-X tubes to the 2ml screw-cap sample tubes. Add 1µl carrier RNA (concentration @ 1µg/µl) to each sample tube.

*NOTE: the addition of 1µl carrier RNA (concentration @ 1µg/µl) is not required for known reference samples.

3. Make sure the DNA Investigator protocol card is fully inserted in the EZ1 and turn the instrument on.
4. Press “Start” to display the “Protocols” menu. Select “Trace TD Protocol” and follow the screen prompts to select the appropriate conditions for the samples being processed and to set up the worktable. Press “Start” to begin the purification procedure.
5. When the protocol ends, “Protocol finished” will be displayed. Retrieve and re-cap the elution tubes.
6. Remove and discard the disposable labware. Follow the cleaning procedure as outlined in the QIAGEN BioRobot EZ1® Workstation Maintenance procedure in this manual. A 20 minute (minimum recommended time) UV decontamination step may be selected following the cleaning procedure.

Note: Due to the chaotropic salts present in the cartridges, do not dispose of them in any bleach or container which may contain bleach as cyanide gas may result.

Samples are ready for human DNA quantitation. Use samples immediately for analysis, otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

NOTE: Prior to quantification, inspect the tubes containing the eluate for the presence of magnetic particles. If magnetic particles are observed, the tube should be applied to a magnetic separator, and the eluate transferred to a clean tube in order to minimize the risk of magnetic particle carryover which may affect the quantification reading.

NOTE: If DNA extracts are likely to be combined with other DNA extracts and/or concentrated using the vacufuge, the samples may be eluted in water, rather than TE. Caution should be used in storing DNA eluted with water since there is no buffering capacity. Extracts in water shall be used the same day or stored frozen until use. If extracts are to be stored at room or refrigerator temperatures, TE or DNASTable must be incorporated.

MICROCON® CONCENTRATION OF DNA

1. Assemble a Microcon® Fast Flow concentrator unit. To the top of the concentrator, add 30-100 µL TE, if desired.
2. Transfer the aqueous phase from the organic extraction procedure (or any DNA extracts to be concentrated) to the top of the concentrator. Avoid pipetting organic solvent from the tube into the concentrator. If using a Microcon to concentrate samples or combine and concentrate samples, add 20 µL of DNA Stable®. The total volume of extract, TE, and DNA Stable® (if used) added to the sample reservoir should be no more than 500 µL.
3. Cap the concentrator and spin in a centrifuge at ~500 to 5000 x g for 10-15 minutes or longer (settings can be adjusted according to centrifuge type and sample viscosity).
4. Carefully remove the concentrator unit from the assembly and discard the filtrate fluid from the filtrate vial. Return the concentrator to the top of the filtrate vial.
5. Add 200-500 µL TE to the concentrator. Recap and spin the assembly in the centrifuge for ~10 minutes at ~500 to 5000 x g.

NOTE: 1) Additional washes may be required to remove inhibitors that may be present and should be done on samples of extremely limited quantity.
2) If the microcon is being used to just concentrate or reduce sample volume, the addition of TE as a wash step is not necessary.

6. Remove the cap, if adding TE, and add the desired final volume of TE (usually between 25 and 200 µL) to the concentrator.
7. Remove the concentrator from the filtrate vial and carefully invert the concentrator onto a new labeled retentate vial. Discard the filtrate vial.
8. Centrifuge the assembly at 1000 x g for 5 minutes.
9. Discard the concentrator. Cap the retentate vial.

Samples are ready for human DNA quantitation. Use samples immediately for analysis, otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

AMICON® ULTRA-4 CONCENTRATION OF DNA

1. Unscrew cap of tube and add between 1.5 and 3.5 ml TE buffer to the filter unit (see figure 1).
2. Transfer the aqueous phase from the organic extraction procedure (or any DNA extracts to be concentrated) to the filter unit to bring the total volume to between 2 and 4 ml.
3. Screw the cap back on the tube and place in a 35 degree fixed angle centrifuge such that the numbers on the filter unit are facing up. That is, the membranes should be facing the sides and the scientist should be able to see the “V” shape of the filter unit as it is placed in the centrifuge.

NOTES: The inner-most ring of the Hermle centrifuges yields the lowest retentate volume. Swinging bucket rotors may also be used, however, the analyst may experience lower retentate volumes.

4. Spin the assembly in the centrifuge for 15 to 20 minutes at 2,000 x g.
5. Uncap each tube, pull out the filter unit, and discard the filtrate at the bottom of the tube. Replace the filter unit into the tube.
6. Add 2 - 4 ml TE buffer to wash, if needed. Spin for 15 to 20 minutes at 2,000 x g. Additional washes may be needed to remove inhibitors.
7. The Amicon® Ultra-4 has a “dead stop” filter unit. The retentate is captured at the bottom of the “V” of the filter unit. Retentate volume should be 30-60 µl.

NOTE: One technique for ensuring maximum volume recovery is as follows: Pull the filter unit from the tube and, while holding it upright, orient it such that the liquid can be seen through the side of the filter unit (the side with no numbers on it). Insert a p-100 pipette tip (fitted onto a pipette set at approximately 60 µl) and pull up the liquid. Monitor the tip to ensure that liquid transfer is taking place. If no liquid is pulled into the tip, swipe the tip across the bottom of the filter unit to push aside any bubble that might be present and try recovery again.

Samples are ready for human DNA quantitation. Use samples immediately for analysis, otherwise, refrigerate or freeze samples for storage while the case is actively being worked (avoid repeated freeze-thaw cycles). Freeze samples for long term storage. Prior to use for PCR, vortex and pulse spin refrigerated and frozen samples.

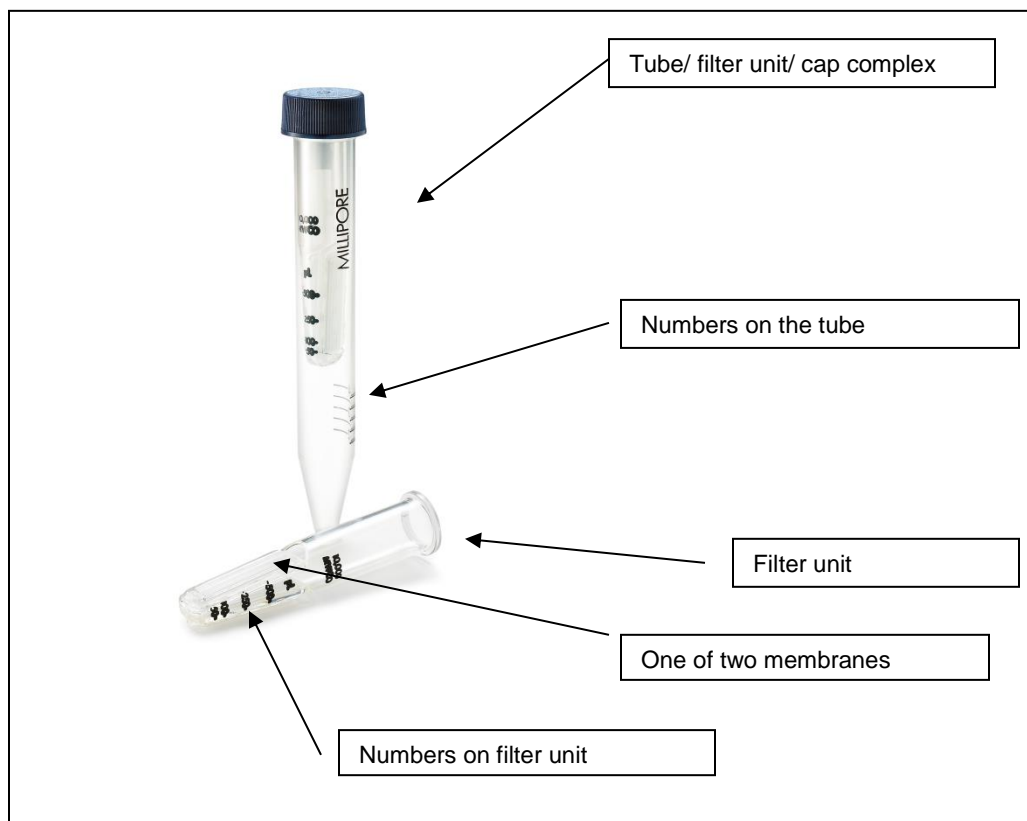


Figure 1: Diagram of Amicon® Ultra-4 Centrifugal Filter Device

VACUFUGE™ PROCEDURE – CONCENTRATION, PRESERVATION, AND RECOVERY OF DNA EXTRACTS/WORK PRODUCT

This procedure can be used to concentrate a sample to a desired volume, combine multiple DNA extracts in a single desired volume, or desiccate a sample to dryness for transfer/return while preserving the integrity of the sample. The addition of DNASTable® prior to concentration/desiccation improves recovery for DNA extracts.

As a general rule for sample concentration/re-hydration, when samples are in TE and concentrated, dH₂O should be used to bring them up to final volume. When samples are in water and concentrated, TE should be used to bring them up to final volume.

When preparing samples for transfer to another laboratory for additional analysis (i.e. Y-STR analysis) or return to the submitting agency, this procedure is used in conjunction with the DNA Extract and Work Product Transfer/Return procedure within this manual. Please refer to that section for further information.

BEFORE AND DURING A RUN

To prevent liquid from condensing in the pump and then later in the lid of the rotor chamber, allow the pump to warm up for 15 minutes prior to the first run of the day. The warm up phase reduces the condensation of liquids in the pump, thus extending the life of the pump. Depending on model, pressing the **Start** button or Mode/Vent button from time to time during the evaporation process allows room air to flow through the pump, causing the collected condensation to move from the pump into the connected separator. Repeat this for several seconds at the end of the last run of the day to remove the remaining condensate from the vacuum pump.

CONCENTRATION OF DNA EXTRACTS

Ensure the correct program is selected for operation (e.g. V-AQ).

1. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
2. Add 20µL of DNASTable® to each DNA extract tube. If multiple samples are to be combined, transfer the samples to a single tube and then add 20µL of DNASTable® to the combined extracts. Mix the sample thoroughly by gentle pipetting; avoid forming air bubbles.

NOTE: For TE-eluted DNA extracts (organic or EZ1), avoid greater than 5X concentration or reduction in volume whether single extract concentration or multiple extract combination followed by concentration.

- If ≤ 5X concentration or multiple extract combination, follow the Vacufuge Procedure. Avoid greater than 5X concentration or reduction in volume whether single extract concentration or multiple extract combination follow by concentration.
- If >5X concentration or multiple extract combination, proceed with a membrane filter device concentration with the addition of DNASTable®

If a membrane filter is used and extract remains after STR amplification that must be sent back to the submitting agency, add an additional 20µL of DNASTable® before drying down in the Vacufuge™.

NOTE: DNASTable concentrations up to 10X have been validated and shown not to adversely affect STR amplification. Increases in DNASTable concentrations may occur when multiple extracts are combined after initially being dried down.

3. Symmetrically load the rotor for balance and ensure that the tubes are open. If possible, leave open spaces between the tubes to be evaporated.
4. Close the lid and press **Start**.
5. Allow the samples to evaporate. Evaporation time is dependent on multiple factors including ambient temperature, humidity, and starting volume of the sample. Samples may be dried to a specific volume or to complete dryness.
 - a. Evaporation/Concentration to a specific volume
 - i. Periodically check the sample to ensure the desired volume.
 - ii. If over-drying has occurred, TE or sterile dH₂O can be added to the sample to bring the volume back to the desired amount. TE will be used if the sample was originally in water and dH₂O will be used if the sample was originally in TE.
 - b. Complete evaporation
 - i. Add sterile dH₂O to the dried sample to achieve the appropriate volume (but not less than 10µL). Optionally, if the sample was stored in water prior to drying, it can be rehydrated with the addition of TE buffer.
 - ii. Incubate at room temperature for a minimum of 15 minutes to allow complete rehydration.
 - iii. Mix the sample with gentle pipetting. Avoid forming bubbles while pipetting.
6. Press **Stop** to release the vacuum and decelerate the rotor. When the rotor stops, the lid will be unlocked.
7. After the samples are removed, wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.

PRESERVATION OF DNA EXTRACTS/WORK PRODUCT FOR TRANSFER/RETURN

This procedure is to be used in conjunction with the DNA Extract and Work Product Transfer/Return procedure within this manual.

1. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
2. Within the case file, record the approximate volume of DNA extract in the tube prior to drying. This step is not required for any remaining cellular work product not subjected to DNA extraction (i.e. cell pellets/substrates).
3. Add 20µL of DNASTABLE® to each DNA extract tube to be dried. Mix the sample thoroughly with gentle pipetting; avoid forming air bubbles. This step should be omitted for other cellular work products and sample cuttings not subjected to DNA extraction.
4. Symmetrically load the rotor for balance and ensure that the tubes are open. If possible, leave open spaces between the tubes to be evaporated.
5. Close the lid and press **Start**.
6. Allow the samples to evaporate to dryness. Evaporation time is dependent on multiple factors including ambient temperature, humidity, and starting volume of the sample. The typical evaporation time is approximately 30-45 minutes to dry a sample completely.

7. Press **Stop** to release the vacuum and decelerate the rotor. When the rotor stops, the lid will be unlocked.
8. Ensure that the sample is completely dry. Completely dried samples should not look sticky or tacky when tapped with a sterile pipette tip. If liquid remains in any sample, return it to the Vacufuge and repeat the drying process to complete desiccation.
9. After the samples are removed, wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
10. Place the preserved DNA extract/work product tubes in a heat-sealable foil pouch or plastic bag with a desiccant pack and heat seal the pouch. Samples are now ready for transfer/return.

Note: For optimal protection during the drying process, do not exceed 30µg of DNA per tube. Prolonged exposure to light may cause fading or color change of DNASTable; however, this will not affect the protective properties of the matrix.

RECOVERY OF DNA EXTRACTS

Preserved DNA extracts and the associated reagent blank(s) submitted for analysis will be recovered following this procedure. If the reagent blank has been retained at the laboratory, it will be retrieved and recovered following this process.

1. Add sterile dH₂O to the dried sample to return it to the previously recorded volume or appropriate volume (but not less than 10µL). Optionally, if the sample was stored in water prior to drying, it can be rehydrated with the addition of TE buffer.

Note: Re-quantification of the samples should be considered prior to amplification.

2. Incubate at room temperature for a minimum of 15 minutes to allow complete rehydration.
3. Mix the sample with gentle pipetting. Avoid forming bubbles while pipetting.

Samples are now ready for human DNA quantitation. Unused rehydrated samples should be stored in the refrigerator or can be maintained at room temperature for up to 10 days. The rehydrated samples still contain DNASTable and can be re-dried without loss of sample stabilization. It is not recommended to repeat the drying/rehydrating process more than 3 times.

DNA QUANTIFICATION: PLEXOR® HY STANDARDS PREPARATION

DNA quantification standards are critical for accurate analysis of run data. Any mistakes or inaccuracies in making the dilutions directly affect the quality of the results. Great care shall be taken when measuring and mixing dilutions. DNA quantification standards shall be run in duplicate for each plate.

The Plexor® HY System is supplied with the Plexor® HY Male Genomic DNA Standard at 50ng/μl. This DNA is a mixture of human male DNA. Store the DNA standard at 4°C overnight before using it for the first time and vortex prior to use. After initial thawing, store at -4°C. Avoid multiple freeze-thaw cycles of the Plexor® HY Male Genomic DNA Standard; variability in the standard curve may increase with multiple freeze-thaw cycles.

1. Prepare the quantification standards using the Plexor® HY Genomic DNA Standard provided in the Plexor® HY kit.
2. Label seven disposable tubes to be used for the dilution series and one tube for the No Template Control (NTC).
3. Prepare TE/glycogen by adding 1μl glycogen to 1 ml of TE buffer. Vortex.
4. Prepare the standards according to the example table below. Use a new pipette tip for each transfer in the series. Vortex and pulse spin each dilution prior to each transfer in the series.

Standard	Conc. (ng/μl)	Calculated Amounts *		Dilution Factor
		DNA †	TE/glycogen	
Std. 1	50.0	100μl undiluted Male Genomic Standard	--	1X
Std. 2	10.0	25μl undiluted Male Genomic Standard	100μl	5X
Std. 3	2.0	25μl [Std. 2]	100μl	5X
Std. 4	0.40	25μl [Std. 3]	100μl	5X
Std. 5	0.080	25μl [Std. 4]	100μl	5X
Std. 6	0.016	25μl [Std. 5]	100μl	5X
Std. 7	0.0032	25μl [Std. 6]	100μl	5X
NTC	--	--	100μl	--

* Reference Plexor® HY System Technical Manual for minimum amount preparations. Prepare standards using TE/glycogen.

† To ensure the accuracy of pipetting, the minimum input volume of DNA for dilutions is 10 μl.

The prepared Male Genomic DNA Standard dilution series may be stored for up to one month at 2 to 8°C.

DNA QUANTIFICATION: PLEXOR® HY REACTION PREPARATION

The amount of PCR-amplifiable human and male DNA is simultaneously determined using the Plexor® HY DNA kit. This assay is performed using the ABI Prism® 7500 Sequence Detection System (SDS).

NOTE:

- Standards shall be run in duplicate.
 - Gloves shall be worn when handling kits and kit components.
1. Determine the number of reactions to be set up. This should include negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. It is critical that the same reaction mix is used for the entire run.
 2. Calculate the volume of the Plexor® HY 2X Master Mix needed to prepare the reaction master mix (10µL per reaction including samples and standards). Calculate the volume of Amplification Grade Water needed to prepare the reaction master mix (7µL per reaction including samples and standards). Calculate the volume of Plexor® HY 20X Primer/IPC Mix to prepare the reaction master mix (1µL per reaction including samples and standards).
 3. Thaw the Plexor® HY 2X Master Mix, Plexor® HY 20X Primer/IPC Mix and Amplification Grade Water.

Note: Do not thaw the Plexor® HY 2X Master Mix and Plexor® HY 20X Primer/IPC Mix at temperatures above room temperature.

4. Briefly vortex the Plexor® HY 2X Master Mix and Plexor® HY 20X Primer/IPC Mix for 3-5 seconds to mix. Do not centrifuge after vortexing, as this may cause the primers to be concentrated at the bottom of the tube.
5. Pipette the calculated volumes of the Plexor® HY kit components into an appropriately sized tube.
6. Vortex briefly to mix.
7. Obtain a 96 well reaction plate and seat in the optical support base. The reaction plate shall not be rested on any surface without the base. The plate shall not be touched on the bottom of the wells.
8. Dispense 18µL of the reaction master mix into each reaction well of the reaction plate.
9. Add 2µL sample, standard, or control to the appropriate well of the reaction plate, as determined by the plate setup sheet.
10. Seal the reaction plate with the Optical Adhesive Cover. Care shall be taken not to touch the adhesive cover. The plastic applicator shall be used to seal the adhesive. Drag the applicator across the adhesive cover several times to ensure a proper seal. Seal around the edges and remove the white strips on the adhesive cover.
11. Shake the plate and centrifuge as necessary to eliminate bubbles.
12. Open the ABI Prism® 7500 instrument and place the 96 well plate in the ABI Prism® 7500 with well A1 in the upper left corner. The tray slides out for loading and pushes back in for the run.
13. Create a plate document from a template

- a. In the SDS software, open a new document.
- b. The settings in the dialog box should read **Assay**: Absolute Quantification (Standard Curve) and **Container**: 96-Well Clear. For Template, select an appropriate template for the list. If the template is not in the list, click Browse to locate the required template (i.e. this may also be an Excel template filled out in advance in Excel with the test sample info). Select “Finish”.
- c. Ensure the following:
 - i. Setup tab: The detectors are correct for the assay. Select “View” from the menu then “Well Inspector”
 - ii. Instrument tab: Thermal cycler protocol and sample volume are correct.
- d. Save the plate document as a *.sds file with an appropriate name
- e. Under the instrument tab, press “Start”.

NOTE: Don't omit a well before the data has been collected.

14. After the run is completed, remove the 96 well plate and discard it in the biological waste.
15. Turn off the ABI Prism® 7500 instrument.

PLEXOR® HY – PRELIMINARY DATA ANALYSIS AND DATA EXPORT

Before data can be analyzed using the Plexor® Analysis Software, the raw data must be analyzed in the SDS software, then exported. Two files must be exported: amplification run data (Delta Rn) and melt/dissociation data (Dissociation). When naming the files, use a descriptive name (e.g. amp or melt) to distinguish the amplification data file from the dissociation data file while maintaining that the files are related.

1. In the SDS software, highlight all unused wells. Go to the View menu and select “Well Inspector” (or enter keyboard option Ctrl+1) to open the Well Inspector window. Select “Omit Well”.
2. Go to the Analysis menu and select “Analysis Settings”, ensure “Automatic Baseline” is selected.
3. Analyze the data by selecting the green arrow icon. The raw data must be analyzed using the SDS software prior to export.
4. To export the amplification data, select “File”, “Export”, then “Delta Rn”. Save this .csv file with an appropriate identity and descriptor (e.g. mm-dd-yy amp.csv) in an appropriate location for import to the Plexor® Analysis Software.
5. To export the melt/dissociation data, select “File”, “Export”, then “Dissociation”, then “Raw and Derivative Data”. Save this .csv file with the appropriate identity and descriptor (e.g. mm-dd-yy melt.csv) in an appropriate location for import to the Plexor® Analysis Software.

Note: When a dissociation curve is included in a thermal cycling program, the SDS software may expect SYBR® green as the dye choice. A caution message may appear to indicate this. Select “Yes” and continue.

PLEXOR® HY SYSTEM DATA ANALYSIS AND INTERPRETATION

The Promega Plexor® HY System Real-Time PCR assay using the ABI Prism® 7500 Sequence Detection System (SDS) is used to simultaneously determine the amount of PCR amplifiable human and male DNA. The Internal PCR Control (IPC) in the assay monitors for the presence of PCR inhibitors and may indicate when it is appropriate to consider additional DNA sample clean up steps.

After data import is complete, it is displayed in the PCR Curves tab of the Analysis Desktop. Three additional tabs (Sample IDs, Standard Curves, and Reports) will be utilized for viewing data analysis metrics. The Forensics Report tab provides concise information regarding IPC status and Curves Status (T_m).

Data Import into Plexor® Analysis Software

1. Launch the Plexor® Analysis Software and select a new run. Select “Next”.
2. Enter operator name and optional additional information.
3. Browse to locate the appropriate exported amplification and melt (dissociation) data files. Select “Finish”.

Plexor® HY Data Analysis

1. Define the DNA standards
 - a. Highlight the wells that contain the DNA standards.
 - b. Select the Create Dilution Series icon. Select “Vertical Series”. Enter 50 for the Starting Concentration and 5 for the Dilution Factor. Select “Decreasing”. These entries will typically remain set after first use.
 - c. Click Apply.
2. Define the No Template Controls (NTC)
 - a. Highlight the wells that contain the NTCs. (Reagent blanks may be designated as NTC – see Optional step below.)
 - b. Select the NTC icon.
3. Assign sample names to the unknowns
 - a. The sample names can be copied from the plate setup template (e.g. DNA Quantification Plate). The layout of the samples names in the template must be the same as the layout of the samples in the PCR plate.
 - i. Highlight all sample names in the plate setup template and select “Copy”.
 - ii. In the Plexor® Analysis Software, select the “Sample IDs” tab.
 - iii. In the Edit menu, select “Paste Sample IDs from Template” or ctrl +T.
4. **Optional:** Define other sample designations
 - a. Reagent blanks may be designated as NTC. The software will “flag” a quant result in an NTC-designated sample with “Check NTC” in the Curves Status of the Forensics Report.
 - b. Colors can be assigned to samples to provide distinction to the displayed data. Select “Color Assignment” to apply a color to selected sample(s).
5. Adjust the Expected Target Melt Temperature. The Plexor® technology allows the use of a melt curve or dissociation curve to determine the melting temperature (T_m) of the products following amplification. This is useful in assessing the specificity of the reaction.
 - a. The expected melt temperature range must be adjusted for all three dye channels (FL, CO560, CR610).
 - b. Select the PCR Curves tab.

- c. Select wells containing the DNA standards.
 - d. In the melt curves window, select the numbered line (expected target melt temperature) of the range window (target melt temperature range) and drag to the midpoint of the melt curves.
6. Generate a Standard Curve.
 - a. In the autosomal channel (FL - Autosomal tab), select all samples and DNA standards. Select “Add Standard Curve” to generate a standard curve and determine DNA concentrations of the unknowns based on the standard curve.
 - b. Repeat for the Y channel (CO560 - Y tab).

Plexor® HY Data Interpretation

Set the Forensics Report to display results without normalization of volumes. Select “Set Normalization and IPC Parameters” in the Forensics menu. Check the box to “...disable volume normalization (show concentration and Ct values only)”.

1. Standard Curve Evaluation (efficiency of reaction) – Standard Curves tab

The slope of the standard curve should be within the typical range as established by internal validation (see table below).

Autosomal slope range	Y slope range
-3.79 to -3.16	-3.73 to -2.97

The standard curve is run in duplicate for precision purposes; however, only one acceptable standard curve is required for analysis. If one of the two duplicate standard curve sets is not acceptable, that set may be entirely removed and the remaining acceptable standard curve set may be used. One of the duplicates in a standard sample can be removed from the standard curve if it is an outlier. This may be done for up to two of the standard samples as long as there is one sample from the duplicate set that is acceptable. One or both of the lowest standards (0.0032ng/μl) may be removed from the standard curve to achieve the acceptable slope range(s). Removing a standard from the curve set(s) is done by deleting the selected well(s) or by changing the designation from “standard” to “unknown”. Changes to the standard curve shall be documented in the case file.

If the slope is outside the determined range after the above alterations have been made, the quantification is not optimal. See **NOTE** below.

2. R² (correlation coefficient) Evaluation - Standard Curves tab

The R² indicates the statistical significance of the standard curve. The R² value should be 0.990 or greater. If a low R² value is observed, change the sample type for one or both of the lowest standards (0.0032ng/μl) from “standard” to “unknown”.

If an R² value of 0.990 or greater cannot be achieved, the quantification is not optimal. See **NOTE** below.

3. Melt Temp Evaluation (assessment of specificity) – multiple tabs

The melt threshold (T_m) is the level of signal that must be reached for the Plexor® Analysis Software to “call” the melt results indicated by a “Yes”, “No”, or “No Call” in the T_m ? column.

“Yes” or “No” in the column indicates whether a sample T_m is within the expected target melt temperature range. A “No Call” in the column indicates that the melt curve displays the expected target melt temperature, but there is insufficient amplification product to cause the melt curve to cross the melt threshold.

A “Yes” for a sample is expected to yield a normal DNA quantification amplification.

“No Call” in the column indicates that the melt curve displays the expected target melt temperature, but there is insufficient amplification product to cause the melt curve to cross the melt threshold.

“No” in the column indicates that there is no T_m for the sample or the sample T_m falls outside the expected target melt temperature range, and the result is due to no DNA (no value in the C_t column) or a false positive.

4. IPC Evaluation – Reports - Forensics tab

The Internal PCR Control (IPC) provides an indication of potential inhibition that may have affected the quantified data.

A display of “Check IPC” in the IPC Status of the Forensics Report indicates that the IPC C_t value in a sample differs from the IPC C_t value in the DNA standard by more than 2 cycles. This result could be due to the presence of a PCR inhibitor and may mean the amount of DNA indicated could be greater and subsequent STR analysis could be affected. DNA concentrations in excess of 10ng/μl could also cause a “Check IPC” display.

Sample clean-up and re-quantification may be appropriate (see appropriate procedures).

5. Quantification Results Evaluation for input into STR Reactions

- a. Using the Samples Details Report or PCR curves tabs, view the C_t values. Samples that do not cross the amplification threshold are listed as not having a valid C_t value. If low DNA quantities result from quantification, additional casework approaches to increase the amount of target DNA available may be considered. Due to the potential unreliability of extremely low level data, samples with target DNA less than 100pg of total DNA shall not be amplified. (See [Auto]/[Y] ratio guidelines below.) If multiple sample extracts with similar probative value are quantified (e.g. sexual assault evidence collection kit swabs), the analyst may choose which sample or samples (if any) to amplify based on case approach considerations, which may include discussions with the submitting agency and/or prosecutor. If sample extracts have been quantified and the submitting agency and/or prosecutor communicates to the analyst that no further examination is needed, the analyst does not need to amplify these extracts. The reasons for stopping analysis must be documented in the case file notes and reported to the submitting agency.

- b. In the Forensic Report table, Curves Status “OK” indicates the following
 - i. the sample, if defined as a standard, shows amplification
 - ii. the sample, if defined as a no-template control, shows no amplification
 - iii. if a melt peak is present, the T_m is within the expected range.

If these criteria are not met, “Check STD”, “Check NTC”, or “Check Melts” will be displayed if these criteria are not met.

c. [Auto]/[Y] Ratio Evaluation

The Plexor® Analysis Software provides a calculated ratio of total human autosomal DNA content to male DNA content. The ratio is used to suggest when the sample may benefit from Y-STR analysis in addition to, or instead of, autosomal STR analysis to produce a meaningful STR profile.

[Auto]: Concentration of total human autosomal DNA in a sample in ng/μl

[Y]: Concentration of human male DNA in a sample in ng/μl

[Auto]/[Y]: Ratio of total human autosomal DNA concentration to male (Y) DNA concentration. A very high [Auto]/[Y] ratio is indicative of a “male/female” mixture with minimal male DNA. The [Auto]/[Y] ratio is NOT intended to identify minor female contribution in a male sample.

To decrease the risk of obtaining insufficient genetic information from the male component of a male/female mixture where there is limited male DNA available, the following table offers amplification guidelines based on the [Auto]/[Y] ratio obtained.

[Auto]/[Y] ratio	Amplification Approach	
	Limited Male DNA	Substantial Male DNA
≤10:1	A	A
10:1 – 100:1	Y	A and Y*
>100:1	Y	Y

A – Autosomal STR

Y – Y-STR

* Typing results at the 3 Y-STR loci included in the Fusion 6C multiplex may be considered for expected interpretable Y results before proceeding to Y-STR amplification

Due to some individual-to-individual variation in copy number of the autosomal target and Y-chromosome target, a single male DNA may not have an [Auto]/[Y] value of 1.0. The internal validation range observed for all laboratories was 0.41 to 3.89 with most samples distributed within the range of 0.4 to 2.0. This is consistent with the developmental validation [Auto]/[Y] ratio distribution within the range of 0.4 to 2.0 for 88% of the samples. For a male contributor displaying low Y-chromosome copy number, targeting more DNA may be considered to increase the chance of obtaining more genetic information.

- d. View all no DNA template samples (reagent blanks, NTCs, etc.). If a DNA quantity value is present for a negative sample, check the melt curves to see if a possible false positive result was obtained.
- e. If a negative DNA quantity result is obtained for a sample, the analysis should be repeated for confirmation. Additional casework approaches to increase the amount of target DNA available may be considered prior to re-quantification.

NOTE:

In the instance that quantification is not optimal (based on either standard curve, R2, or both), the following should be checked:

- Plexor® HY assay setup including Plexor® HY reagents
 - Quantification standards load verification
 - Preparation of the quantification standards
- 7500 instrument and corresponding computer setup
- SDS software setup
- Plexor® HY Data Analysis setup

Quantification results from a non-optimal Plexor® HY assay may still be used, but only to provide limited assistance in making analytical decisions (i.e. approximating the dilution to use for the desired target amount of DNA to amplify).

DNA QUANTIFICATION: POWERQUANT™ SYSTEM STANDARDS PREPARATION

PowerQuant™ System

The PowerQuant™ System is supplied with the PowerQuant™ Male gDNA Standard at 50ng/μl. Store the DNA standard at 4°C overnight before using it for the first time and vortex prior to use. After initial thawing, store at -4°C. DO NOT refreeze the Male gDNA Standard; variability in the standard curve may increase with freeze-thaw cycles.

1. Prepare the quantification standards using the Male gDNA Standard provided in the PowerQuant™ kit.
2. Label four (or seven) tubes to be used for the dilution series and one tube for the No Template Control (NTC).
3. Prepare the standards according to the example tables below. Use a new pipette tip for each transfer in the series. Vortex and spin each dilution prior to each transfer in the series.
 - a. The Dilution Buffer provided in the PowerQuant™ kit or TE/glycogen may be used to dilute the standard series. Prepare TE/glycogen by adding 1μl glycogen to 1ml of TE buffer. Vortex.

7-Point Standard Curve (Fivefold Dilutions)

Standard	Conc. (ng/μl)	Calculated Amounts *		Dilution Factor
		Male gDNA	Dilution Buffer (or TE/glycogen)	
Std 1	50	50μl Male gDNA (undiluted)	--	--
Std 2	10	25μl Male gDNA (undiluted)	100μl	5X
Std 3	2	25μl std. B	100μl	5X
Std 4	0.4	25μl std. C	100μl	5X
Std 5	0.08	25μl std. D	100μl	5X
Std 6	0.016	25μl std. E	100μl	5X
Std 7	0.0032	25μl std. F	100μl	5X
NTC	--	--	100μl	--

4-Point Standard Curve (25-Fold Dilutions)

Standard	Conc. (ng/μl)	Calculated Amounts		Dilution Factor
		Male gDNA	Dilution Buffer (or TE/glycogen)	
Std 4-1 [Std 1]	50	50μl Male gDNA (undiluted)	--	--
Std 4-2 [Std 3]	2	4μl Male gDNA (undiluted)	96μl	25X
Std 4-3 [Std 5]	0.08	4μl std. B	96μl	25X
Std 4-4 [Std 7]	0.0032	4μl std. C	96μl	25X
NTC	--	--	100μl	--

* Reference PowerQuant™ Technical Manual for minimum amount preparations.

The prepared Male gDNA Standard dilution series may be stored for up to one month at 2 to 8°C.

When necessary (i.e. a manual plate load), the appropriate Standard dilution tubes (1, 3, 5, 7 and NTC) can be pulled from the 7-point standard curve to “create” a 4-point curve. The 7-point standard curve lot number shall be sufficient documentation in the case notes.

The NTC has been included in the Standard dilution series however, it may be excluded from the set and amplification grade water provided with the PowerQuant™ kit may be loaded to the NTC wells.

DNA QUANTIFICATION: POWERQUANT™ SYSTEM REACTION PREPARATION

The amount of PCR-amplifiable human and male DNA is simultaneously determined using the PowerQuant™ System. This assay is performed using the ABI Prism® 7500 Sequence Detection System (SDS).

NOTE:

- Standards shall be run in duplicate.
- Gloves shall be worn when handling kits and kit components.
- Artifacts may occur if the time between amplification setup and the start of thermal cycling exceeds 2 hours.

1. Prepare the components of the PowerQuant™ System
 - a. At first use, thaw the 2X Master Mix, the 20X Primer/Probe/IPC Mix, and the Amplification Grade Water. After first use, store at 2-10°C.
 - b. Centrifuge the 2X Master Mix and 20X Primer/Probe/IPC briefly to bring the contents to the bottom THEN vortex the reagents for 10-15sec to mix. (Do not centrifuge after mixing as this may cause a concentration gradient.)
2. Determine the number of reactions to be set up (# of samples = n). The number includes standards, NTCs, DNA samples, and their associated controls. Include additional reactions to compensate for volume loss during pipetting (e.g., n+2). It is critical that the same reaction mix is used for the entire run.
 - i. Water, amplification grade: 7µl x (n+2)
 - ii. 2X Master Mix 10µl x (n+2)
 - iii. 20X Primer/Probe/IPC Mix: 1µl x (n+2)
3. Combine the determined volumes of water, master mix, and primer mix in an appropriate tube and vortex. Do not centrifuge after mixing.
4. Distribute 18µl of reaction mix to the 96 well reaction plate seated in a support base.
5. Add 2µl sample, standard, or control to the appropriate wells of the reaction plate, as determined by the plate setup sheet.
6. Seal the reaction plate with the optical adhesive cover. Ensure that all wells are adequately sealed to prevent evaporation during thermal cycling.
7. Eliminate bubbles in the wells as necessary (centrifuge, etc.).

DNA QUANTIFICATION: POWERQUANT™ SYSTEM STARTING A RUN

1. In the 7500 Software, create an experiment document by opening an appropriate template (.edf file) (ensure the correct standard curve is represented: 7-point or 4-point)
 - a. On the home screen, select the "Template" button
 - b. Navigate to and open the appropriate PowerQuant™ template file
2. Add sample names by selecting the File menu at the top of the screen then select import. Browse to and select the appropriate plate setup file and click "Start Import". Verify that the correct data was imported.

3. Check that “PQ_CXR” is selected as the passive reference.
4. Save the file as a .eds file to an appropriate location.
5. Open the AB 7500 instrument tray door and place the prepared 96 well plate in the plate holder with well A1 in the upper left corner. Push the tray door closed and press “Start Run”. Run time is 1 hour.

NOTE: The unused wells within a prepared plate may be selected and assigned targets (Auto, Degradation, IPC, and Y). This ensures that all well data is captured if the plate had been loaded incorrectly on the 7500 instrument.

DNA QUANTIFICATION: POWERQUANT™ SYSTEM DATA ANALYSIS

Analyze Data and Export

1. In the 7500 Software, select “Analysis” in the left side panel and ensure that all wells to be analyzed are highlighted on the *View Plate Layout* tab and that no targets are selected for the unused wells.
2. Select “Analyze” button above the plate layout. If desired, the standard curves can be reviewed by selecting “Standard Curve” from the *Analysis* menu in the left side panel.
3. Ensure that all wells with data for export are highlighted in the plate map. Select “Export” from the toolbar.
4. On the *Export Properties* tab of the *Export Data* window, select the following:
 - a. Choose “Results” in the *Select data to export* section.
 - b. Choose “One File” from the *Select one file or separate files* drop-down menu.
 - c. Select export file properties: name the file, choose “.xls” as file type, and use the Browse button to select an appropriate file location.
 - d. Select “Start Export”.
5. Select “Close Export Tool” in the *Export Completed* window.

DNA QUANTIFICATION: USING THE POWERQUANT™ ANALYSIS TOOL

The PowerQuant™ Analysis Tool is a Microsoft Excel® macro-enabled template which evaluates and calculates the following:

- Standard curves (acceptable R^2 and slope values)
- Sample quality (possible inhibition, mixture, or degradation)
- DNA normalization for autosomal or Y-STR amplification based on DNA target mass, pipetting volumes and diluent volumes
- Samples that may need additional attention based on validation parameters

The PowerQuant™ Analysis Tool requires the inclusion of sample names in the Results Excel file exported from the AB 7500 software for the calculations.

On first use of the PowerQuant™ Analysis Tool, ensure that the macros have been enabled. Upon opening, the Home worksheet is visible. The other worksheet is the Admin Settings worksheet. Verify the data in the Admin Settings worksheet is as follows:

- Autosomal, Degradation, IPC and Y are the listed target names in table 1.
 - R^2 values for each listed target is 0.99; slope values for each listed target is -3.6 to -3.1 in table 2.
 - Under sample assessment (table 3), the IPC shift value is 0.30, the [Auto]/[Y] ratio is 2.0, and the [Auto]/[D] ratio is 2.0.
 - The Normalization tables may be modified according to scientist needs.
1. Open the PowerQuant™ Analysis Tool. Select the *Import Data* button. Browse to the appropriate exported .xls file, highlight the file and select "OK".

Numerous worksheets are created with tabs at the bottom of the template.

- Standards: Displays the standard curve results for each target and indicates whether the R^2 value and slope meet the criteria defined on the *Admin Settings* worksheet.
- Standard Curves: Displays a plot of the C_q value versus DNA concentration for each quantification target and the equation line of best fit.
- Results: Displays the data output sorted by well position. The *Instrument Target Name* and *Sample Assessment* settings from the *Admin Settings* worksheet are displayed at the top of the page. The ratios of [Auto]/[Y] and [Auto]/[D] are calculated and displayed on this worksheet.
- Results with Averages: Contains output results sorted alphabetically by sample name, with averages calculated for replicate samples.

DNA QUANTIFICATION: POWERQUANT™ SYSTEM DATA INTERPRETATION

The PowerQuant™ System provides information on the concentration of autosomal and male DNA and allows the user to evaluate amplification performance, detect PCR inhibitors, detect the presence of male and female DNA mixtures, and evaluate the degree of DNA degradation.

The 7500 Real-Time PCR Software and the PowerQuant™ Analysis Tool both perform a linear regression to the standard dilution series data and calculate the equation for the line of best fit (the standard curve).

1. **Standard Curve Evaluation:** The slope of the standard curve is an indication of the PCR efficiency. The R^2 value is a measure of the fit of the data points on the linear regression of the dilution standards.

In general, the average slope of the standard curve for each target (autosomal, degradation, and Y) is in the range of -3.6 to -3.1 with an R^2 value > 0.990.

If the slope of the standard curve is outside the determined range or a low R^2 value is observed, the following adjustments to the standard curve can be attempted. Changes to the standard curve shall be documented in the case file.

- If one of the two duplicate standard curve sets is not acceptable, that set may be entirely removed and the remaining acceptable standard curve set may be used.
- Alternatively, when only one point on the standard curve is an outlier, that one standard point may be removed.

If the slope or R^2 is outside the determined range after any above alteration has been made, the quantification is not optimal. See **NOTE** below.

2. **IPC Evaluation:** The Internal PCR Control (IPC) provides an indication of potential inhibition that may have affected the quantified data.
 - If a sample yields no detectable amplification for the autosomal, Y, and degradation targets but the amplification curve of the IPC crosses the amp threshold without IPC shift greater than 0.3, then insufficient DNA template was added to the amplification.
 - IPC shift > 0.3 or if the C_q value for the IPC is undetermined, an inhibitor may be present.
 - IPC shift > 0.3 and [Auto]/[D] ratio < 2.0, then the sample likely contains a PCR inhibitor but the DNA is not likely degraded.
 - IPC shift > 0.3 and Auto]/[D] ratio > 2.0, then the sample likely contains a PCR inhibitor and the DNA is possibly degraded.

If an unsuitable IPC result is obtained, sample clean-up or re-quantification may be appropriate (see appropriate procedures).

3. **Evaluation of results for input into STR Reactions**

[Auto]: Concentration of total human autosomal DNA in a sample in ng/μl

[Y]: Concentration of human male DNA in a sample in ng/μl

[Auto]/[Y]: Ratio of total human autosomal DNA concentration to male (Y) DNA concentration. It is NOT intended to identify minor female contribution in a male sample.

[Auto]/[D]: Ratio to evaluate whether a DNA sample was degraded.

If low DNA quantities result from quantification, additional casework approaches to increase the amount of target DNA available may be considered. Due to the potential unreliability of extremely low level data, samples with target DNA less than 100pg of total DNA shall not be amplified. If multiple sample extracts with similar probative value are quantified (e.g. sexual assault evidence collection kit swabs), the analyst may choose which sample or samples (if any) to amplify based on case approach considerations. The reasons for stopping analysis must be documented in the case file notes and reported to the submitting agency.

View all no DNA template samples (reagent blanks, NTCs, etc.). Detection of > 1.0pg per 2µl input volume DNA can indicate the presence of contaminating DNA.

If a negative DNA quantity result is obtained for a sample (i.e. no result for both Auto and Y targets), the analysis should be repeated for confirmation. Additional casework approaches to increase the amount of target DNA available may be considered prior to re-quantification.

- a. [Auto]/[Y] Ratio: The ratio is used to suggest when the sample may benefit from Y-STR analysis in addition to, or instead of, autosomal STR analysis to produce a meaningful STR profile.
- No [Auto]/[Y] ratio value, then no male DNA was detected
 - [Auto]/[Y] ratio value < 2.0, then the sample may contain male DNA only or low levels of female DNA.
 - [Auto]/[Y] ratio value > 2.0, then the sample contains a possible mixture of male and female DNA. A very high [Auto]/[Y] ratio is indicative of a “male/female” mixture with minimal male DNA. (See [Auto]/[Y] ratio guidelines below.)

To decrease the risk of obtaining insufficient genetic information from the male component of a male/female mixture where there is limited male DNA available, the following table offers amplification guidelines based on the [Auto]/[Y] ratio obtained.

Amplification Approach		
[Auto]/[Y] ratio	Limited Male DNA	Substantial Male DNA
≤10:1	A	A
10:1 – 50:1	Y	A and Y*
>50:1	Y	Y

A – Autosomal STR

Y – Y-STR

* Typing results at the 3 Y-STR loci included in the Fusion 6C multiplex may be considered for expected interpretable Y results before proceeding to Y-STR amplification

- b. [Auto]/[D] Ratio: The ratio is used to evaluate whether a sample was degraded and can be interpreted as follows.
- [Auto]/[D] ratio < 2.0, then the DNA in the sample is not likely degraded regardless of the IPC shift value.
 - [Auto]/[D] ratio > 2.0 AND the IPC shift < 0.3, then the DNA in the sample is likely degraded but does not contain PCR inhibitors.
 - [Auto]/[D] ratio > 2.0 AND the IPC shift > 0.3, then the DNA in the sample likely contains PCR inhibitors and may or may not contain degraded DNA.
 - [Auto]/[D] ratio is undetermined and IPC shift < 0.3, then the DNA in the sample is likely severely degraded but does not contain PCR inhibitors
 - [Auto]/[D] ratio is undetermined and the IPC shift > 0.3, then the sample likely contains PCR inhibitors and the DNA may be degraded.

Based on validation studies, it may be necessary to increase the amount of target DNA for amplification in

- Fusion 6C: when the [Auto]/[D] ratio is greater than 5
- Y23: when the [Auto]/[D] ratio is greater than 10

NOTE:

In the instance that quantification is not optimal (based on slope or R^2), the following should be checked:

- PowerQuant™ assay setup including PowerQuant™ reagents
 - Quantification standards load verification
 - Preparation of the quantification standards
- The appropriate standard dilution series concentrations were entered into the assay setup.
- 7500 instrument and corresponding computer setup
 - Correct passive reference selected
 - Analysis setting are appropriate in the 7500 software
- Examine the volume of liquid in each well of the plate to verify that evaporation did not occur during cycling

Quantification results from a non-optimal PowerQuant™ assay may still be used, but only to provide limited assistance in making analytical decisions (i.e. approximating the dilution to use for the desired target amount of DNA to amplify).

AMPLIFICATION OF STR LOCI: FUSION 6C

Based on quantification results, the target amplification range of template DNA is 100 picograms (pg) up to less than 4ng (based on Plexor HY results) or 5ng (based on PowerQuant™ results) with a standard target of approximately 1ng for single source samples. Amplification of target DNA less than 100pg (validated amplification threshold) is at analyst discretion. Additional casework approaches to maximize the target DNA shall be considered – see Considerations for Low Target DNA Amplification section below.

1. Prepare the DNA samples to be amplified.

Using the DNA quantification results, calculate the volume of test sample needed to provide the desired amount of DNA to amplify. A TE/DNA or amp grade water/DNA mixture of the appropriate DNA concentration may be prepared at this step if desired. Alternatively, the TE or amp grade water and the DNA can be added to the amplification tubes/wells separately. The final volume of sample added to the PCR reaction is 15µL.

2. Prepare the components from the Fusion 6C System amplification kit.
 - a. At first use, thaw the 5X Master Mix, 5X Primer Pair Mix, and amplification grade water completely. After first use, store at 2-10°C.
 - b. Centrifuge the 5X Master Mix and 5X Primer Pair Mix tubes briefly to bring the contents to the bottom THEN vortex the reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing as this may cause a concentration gradient.
 - c. Appropriately dilute the 2800M positive control (+C) DNA with TE buffer to the desired template DNA volume. 2800M is provided at 10ng/µl.
 - d. A negative amplification control (-C) will be run with every amplification. The -C will be prepared using either TE buffer or amp grade water. If a sample(s) to be amplified was normalized using TE and its associated reagent blank was not normalized, a -C prepared with TE shall be included in the amplification. This may serve as the -C or be an additional -C.
3. Determine the number of samples for amplification (# of samples = n). Include additional reactions to compensate for volume loss during pipetting (i.e., n+2).
 - Master Mix 5µL X (n+2)
 - Primer Pair Mix: 5µL X (n+2)
 - (optional amp grade water): 5µL X (n+2) *[use when DNA template volume is 10µL]*

Vortex the master mix and distribute

4. Set up the amplification. The following steps shall be performed in a biological hood or other area designated for PCR setup.
 - a. Prepare the master reaction mix using the volumes determined above. Vortex the master reaction mix and aliquot 10µL (or 15µL) to the PCR reaction tube/well.
 - b. Add 15µl (or 10µL) of prepared DNA, positive control DNA (diluted to 0.1ng/µL), and TE buffer (negative control) to the appropriate tubes/wells.
5. Load the tubes/plate into the thermal cycler and start the appropriate amplification protocol.

CONSIDERATIONS FOR LOW TARGET DNA AMPLIFICATION

- If multiple sample extracts with similar probative value are quantified (e.g. sexual assault evidence collection kit swabs), the analyst may choose which sample or samples (if any) to amplify based on case approach considerations, which may include discussions with the submitting agency and/or prosecutor.
- If sample extracts have been quantified and the submitting agency and/or prosecutor communicates to the analyst that no further examination is needed, the analyst does not need to amplify these extracts. The reasons for stopping analysis must be documented in the case file notes and reported to the submitting agency.
- If required, DNA extracts can be combined and/or concentrated via the Vacufuge Procedure or by using a membrane filter device (i.e. Microcon®).

THERMAL CYCLER PROTOCOL

96°C for 1 minute, then:

96°C for 5 seconds

60°C for 1 minute

for 29 cycles, then:

60°C for 10 minutes

4°C soak

DIRECT AMPLIFICATION OF STR LOCI: FUSION 6C

Direct amplification can be used for buccal or blood reference samples on FTA or non-FTA substrates (i.e. filter paper, fabric, or swabs). PCR setup shall be performed in a biological hood or other area designated for PCR setup. These reactions are highly dependent on sampling, especially for non-uniform samples. For very weak samples or extracts requiring alternative amplification protocols, the analyst should consider quantifying the DNA extract for a standard amplification.

Sample Preparation

1. Place an appropriate size sample in an extraction tube (typically, at least one-quarter of one swab or at least 3 x 3 mm of a stained substrate)
2. Add sufficient SwabSolution™ Reagent to cover the sample (100 - 1000 µL). A reagent blank shall be initiated.
3. Incubate at 70°C for at least 30 minutes. Extracts can be stored for up to 24 months at ~4°C.

PCR Setup

1. Prepare the components of the Fusion 6C amplification kit.
 - a. At first use, thaw the 5X Master Mix, 5X Primer Pair Mix and Amplification Grade Water completely. After first use, store at 2 - 10°C.
 - b. Centrifuge the 5X Master Mix and 5X Primer Pair Mix tubes briefly to bring the contents to the bottom, then vortex the reagents for 15 seconds before each use. Do not centrifuge after vortexing, as this may cause a concentration gradient.
 - c. The 2800M positive control (provided at 10 ng/µL) DNA may be diluted with Amplification Grade Water.

2. Determine the number of reactions to set up (# samples = n), including positive and negative controls. Include additional reactions to compensate for volume loss during pipetting (i.e., n+1)

Component	Volume per Reaction
Water, Amplification Grade	5.5 µL
5X Master Mix	2.5 µL
5X Primer Pair Mix	2.5 µL
Total volume	10.5 µL

3. Vortex the PCR amplification mix for 5-10 seconds, then aliquot 10.5 µL of PCR mix to the reaction tube/well.
4. Add 2 µL of swab extract, positive control DNA (DNA target 10 ng to 2.5 ng), and negative control (amplification grade water or TE⁻⁴ buffer) to the appropriate tubes/wells.

PCR

1. Load the tubes/plate onto a thermal cycler and start the appropriate amplification protocol. Ensure that Max Mode is selected for ramp speed and 12 µL reaction volume is selected.

Thermal Cycling Protocol

96°C for 1 minute, then:

96°C for 5 seconds

60°C for 1 minute

for 26 cycles, then:

60°C for 10 minutes

4°C for ∞

2. Proceed with Amplification Product Preparation: Fusion 6C

AMPLIFICATION PRODUCT PREPARATION: FUSION 6C

Formamide is a teratogen – wear gloves

1. Prepare a master mix of Hi-Di™ formamide and WEN ILS 500.
 - a. (# of samples + 1) x 9.5µl formamide
 - b. (# of samples + 1) x 0.5µl WEN ILS 500

If an injection will cover wells with no samples, add formamide (or formamide+WEN mixture) to the blank wells.

Note: The volume of internal lane standard used in the loading master mix can be adjusted to change the intensity of the size standard peaks based on laboratory preferences.

2. Vortex and spin briefly. If making a stock tube of mixed formamide and size standard, label the tube with the lot number of WEN added along with the date and analyst's initials.
3. Dispense 10µl of formamide+WEN mix into the appropriate wells of the CE plate.
4. Add 1µl allelic ladder or PCR product to each well, being careful to not introduce bubbles.
5. Cover the plate with a septa cover.
6. Spin briefly to remove any bubbles.
7. Denature samples at 95°C for 3 minutes.
8. Chill the samples for 3 minutes (use a freezer plate block, crushed ice, or ice-water bath).
9. Place plate in a plate base and cover with a plate retainer, being mindful of plate alignment.

In addition to using injection times less than validated default injection times for amplicons with excessive peak heights (e.g. causing -A shoulder artifacts or saturation), a smaller amplicon volume between 0.5µl and 1µl can be loaded, or a dilution in either formamide or TE buffer up to 1:10 can be used. If any of these methods or combinations thereof is used for the analyzed sample, it shall be documented in the case file.

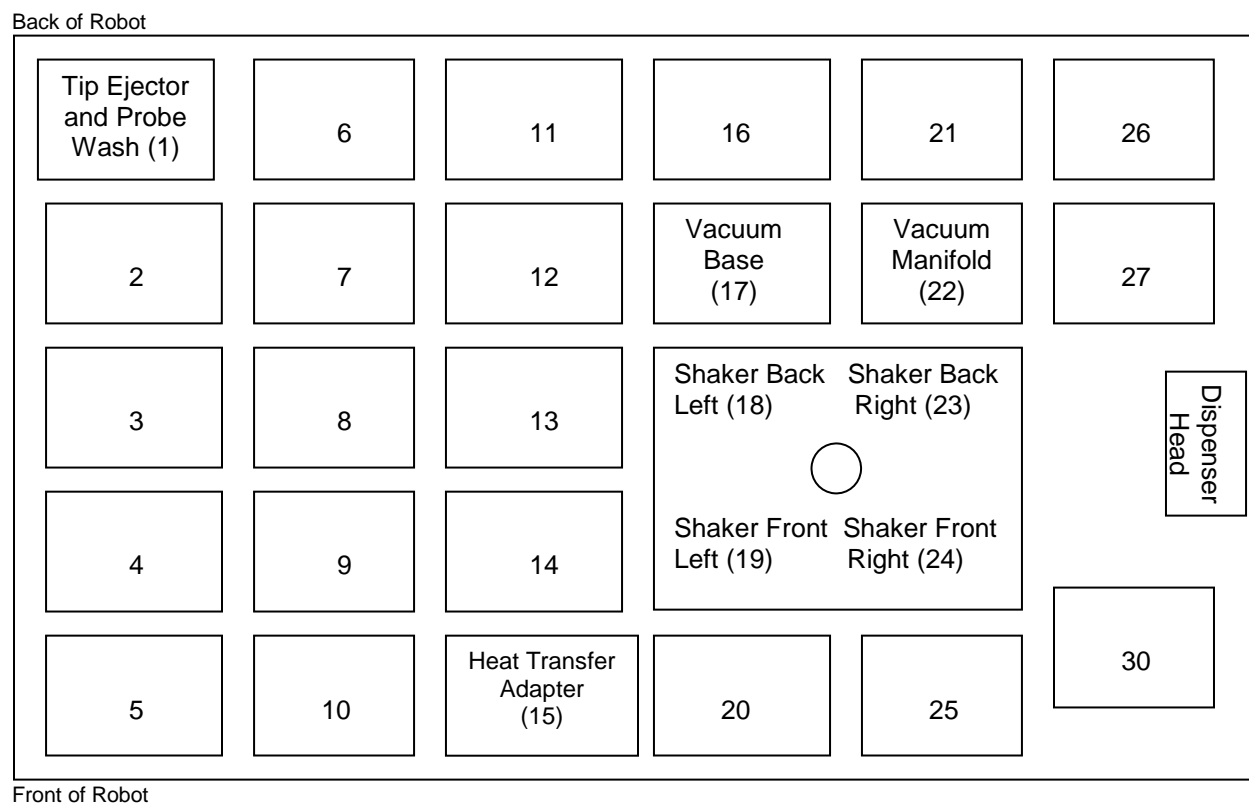
QIAGEN BIOROBOT® UNIVERSAL

INTRODUCTION

The QIAGEN BioRobot® Universal with QIAsoft™ v 5.0 software is used by the Washington State Patrol Crime Laboratory Division laboratories to set up quantitation reactions, prepare quantitation standard dilutions, normalize concentrations of DNA extracts and set up STR amplification reactions.

The layout of the instrument work surface contains 30 positions designated numerically in columns starting from the back left of the instrument (see Figure 1).

Figure 1: Numbering system on the Universal



The QIAsoft™ software for use with the BioRobot® Universal manages the robot and allows development and execution of each protocol. The QIAsoft™ operating system for the BioRobot® contains five protocol environments that are accessible by users based on their user privileges. The Maintenance Environment should be checked before each run to see if a maintenance procedure should be performed. The BioTek® Plate Reader is used in the biannual maintenance of the BioRobot® Universal.

PLEXOR® HY SYSTEM – UNIVERSAL

Preparation of Plexor® HY Standards

1. Turn on the BioRobot® Universal and associated computer. Open the QIAsoft™ software.
2. Under 'PCR Protocols', select the 'Dilution Plexor' protocol. Follow the directions given by the robot. Ensure that Plexor® HY Genomic Standard is being used for Plexor® standards dilution.
3. Following completion of the protocol, cap the tubes and if not proceeding with a quantification, store at ~4°C,

Plexor® HY Quantification Assay Setup

1. Turn on the BioRobot® Universal and associated computer and open the QIAsoft™ Software.
2. Open the 'Quant Setup Plexor' protocol. Follow the directions given by the robot:
 - a. Enter the number of samples (including standards columns).
 - b. Enter the volume of Master Mix to be added per well – 18µl for Plexor® HY.
 - c. Enter the volume of DNA to be added per well – 2µl.
 - d. Prepare Master Mix according to the robot instructions.
3. After the protocol is finished, complete the quantification set up as follows:
 - a. Seal the plate using the Optical Adhesive Cover.
 - b. If necessary, spin plate to remove any bubbles.

Follow the Plexor® HY System Data Analysis and Interpretation SOP for analyzing and interpreting data.

Plexor™ Analysis Software file conversion – Plexor Data Converter

1. In Plexor® Analysis Software, copy the "Forensics Report" data. An error will occur if the appropriate report data is not copied.
2. Open the Plexor Data Converter program. Click the 'Paste Data' button. This will populate two worksheets within the program.
 - a. The 'Forensic Report' worksheet will filter and display quantification data.
 - b. The 'Norm-AMP' worksheet allows for further processing using the QIAgility.
3. Make appropriate changes to the "NORM-AMP" tab. The User Guide may be accessed by clicking the 'Show User Guide' button.
 - a. The 'Process Sample' column designates which samples will be processed:
 - '0' for no process
 - '1' for normalization then amplification
 - '2' for direct amplification (without normalization).
 - b. Other alterations to the table may be made, including 'amp target', 'final volume', and adjustments in the 'name' and 'QTY' columns.
 - i. Amp Target default is 1ng. This value may be changed for the entire set by entering the desired volume in the 'Change Amp Target' area of the converter program. Any individual sample's amp target may be changed as needed.
 - ii. For samples to be directly amplified, amp target will be automatically calculated to reflect input (10µl) by concentration ('Qty' value in converter).
4. Press 'Update' once all appropriate adjustments have been made.
5. Press 'Save Files'. Designate a location for the saved files.

Combined Normalization and Amplification

The robot will normalize designated samples, calculate amplification reaction mix volumes and transfer DNA for normalization and amplification.

Tables created by the "DNA Data Converter" program need to be transferred to the User Data folder of the BioRobot® Universal computer prior to running the protocol.

1. Turn on the BioRobot® Universal and associated computer. Open the QIAsoft™ Software. Select 'PCR Protocols' and open the 'Normalization and Amp' protocol. Follow the directions given by the robot. If an error occurs, check the .csv file and ensure all the values are appropriate.
 - a. Enter the name of the Norm file including the .csv extension.
 - b. Prepare the appropriate amplification reaction mix according to robot instructions.
2. Once the program is complete, cap and store the remaining sample extracts as appropriate.
3. When the run is complete, cap the plate and place on the thermal cycler and start the amplification process.

POWERQUANT™ SYSTEM – UNIVERSAL

Preparation of PowerQuant™ Standards

1. Turn on the BioRobot® Universal and associated computer. Open the QIAsoft™ software.
2. Under 'PCR Protocols', select the 'Dilution Plexor' protocol. Follow the directions given by the robot. Ensure that PowerQuant™ gMale DNA Standard is being used for PowerQuant™ standards dilution.
3. Following completion of the protocol, cap the tubes and if not proceeding with quantification, store at ~4°C.

PowerQuant™ Quantification Assay Setup

1. Turn on the BioRobot® Universal and associated computer and open the QIAsoft™ Software.
2. Open the 'Quant Setup Plexor' protocol. Follow the directions given by the robot:
 - a. Enter the number of samples (including standards columns).
 - b. Prepare Master Mix according to the robot instructions.
2. After the protocol is finished, complete the quantification set up as follows:
 - a. Seal the plate using the Optical Adhesive Cover.
 - b. If necessary, spin plate to remove any bubbles.

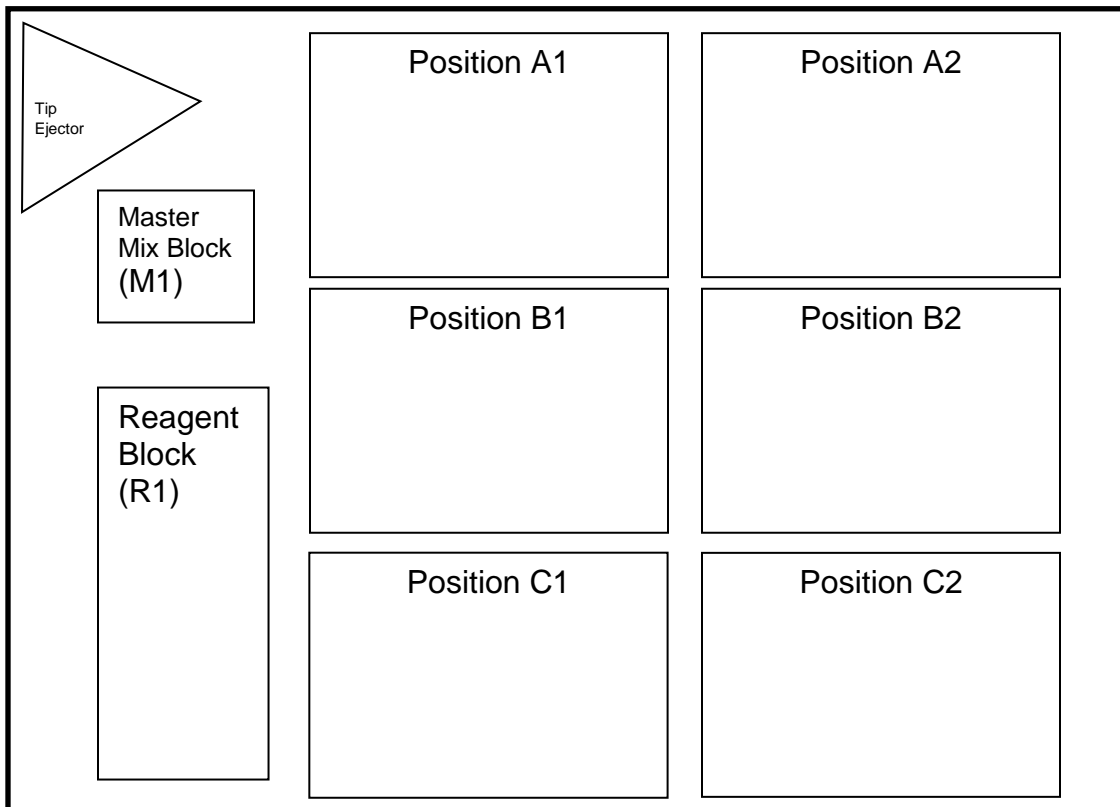
Follow the PowerQuant™ System Data Analysis and Interpretation SOP for analyzing and interpreting data.

QIAGEN QIAGILITY INSTRUCTIONS

INTRODUCTION

The QIAGEN QIagility along with the QIagility Software is used by the Washington State Patrol Crime Laboratory Division laboratories to set up quantification reactions, normalize extracts and set-up amplifications, and set up CE plates for detection.

When using the QIagility, please refer to the protocol in use for proper QIagility deck setup.



QUANTIFICATION SETUP

PowerQuant™ Quantification Assay Setup

1. Enter sample names into the QIagility template sheet of the “4-point PowerQuant™” workbook. This template mirrors the 4 x 8 well racks of the instrument deck. The sample names and load order will automatically be transferred to the sample bank sheet(s) matching the 96-well quantification plate setup and the Plate Template sheet when the “Populate Data” button on the Plate Document sheet is clicked. Save the sample bank sheet(s) and the Plate Document sheets as .txt files.
2. Mix the appropriate volumes of reagents in a 5 mL tube. Place this tube into position C on the Master Mix Block.

3. Place the appropriate DNA Standards and NTC in the proper positions on the QIAgility Deck.
4. Load samples in EZ1 screw cap tubes into the Sample banks racks in the position designated by the QIAgility template sheet. If your samples are in different tubes, be mindful that the instrument is calibrated to the EZ1 screw cap tube, or transfer sample into an EZ1 screw cap elution tube.
5. Place a 96-well optical reaction plate in the appropriate location of the QIAgility Deck.
6. Open the appropriate protocol from the QProtocols folder.
7. In the Software, select/highlight the rack where your samples are located. Click on the “Import” button. Click on the box to the right of the Import File drop-down menu and navigate to the QIAgility.txt file generated above (step 1). Repeat this step as needed if additional sample blocks are in use.

8. Generate a sample bank, making sure the following values are correct:

“From Row:” = 1 (indicates the beginning of the samples to import by skipping header)

“To Well:” = A1

“Limit Sample Count = 64”

“Sample Name = Column 1”

Uncheck the “Load Conc. From Column” box.

Under “Sample Bank Options, select “Add Filtered Rows to Sample Bank” and select “QF Samples” under Existing Bank.

Click the “Import” button, then “Finish.”

In the sample bank window the number following bank “Bank QF Samples” should now correspond to the number of samples in the sample racks.

Note: If a third sample bank is used and this sample is being utilized please make sure the following values are correct:

“From Row:” = 1 (indicates the beginning of the samples to import by skipping header)

“To Well:” = A1

“Limit Sample Count = 32”

“Sample Name = Column 1”

Uncheck the “Load Conc. From Column” box.

Under “Sample Bank Options, select “Add Filtered Rows to Sample Bank” and select “QF Samples-2” under Existing Bank.

Click the “Import” button, then “Finish.”

In the sample bank window the number following bank “Bank QF Samples-2” should now correspond to the number of samples in the sample racks.

Note: If samples appear to have imported incorrectly, the sample bank can be edited by selecting the sample bank from the list in the top right window, clicking on “Edit Bank”, then under Sample Selection of the Update Sample Bank window, click on “Bank Wells” to select all the samples and “Delete Selection.” This should be followed by hitting the “Clear” button above the samples list. Then, re-import your samples following the directions in steps 7-8.

9. On the QIAgility deck map within the software, double-check reaction steps; depending on the protocol in use this can be done by selecting the destination plate. The “Pipette 2µl of QF samples...” reaction listed should be active (formerly grayed out) after populating the QF Samples sample bank. Delete any pipetting function not in use (grayed out).

10. A pre-run report may be used to confirm that tips, tubes, plates and liquids have been set up correctly. It can be accessed by clicking the icon on the toolbar or the Pre-run Report button at the bottom of the checklist window after clicking the green arrow.
11. Click the green arrow at the top to start. When prompted to Save, create a folder with the batch date. The default name for the file can be accepted. Save the file to the newly created folder.
12. Check the boxes in the checklist menu as the actions are completed.

Normalization and Amplification Setup

PowerQuant™ – QIAgility Data Converter

Helpful instructions are also on the “Instructions” sheet of the “WSP QIAgility” workbook.

1. Using the 7500 Software, select all used wells and click “Export”. Export Properties should be:
 - a. Select data to export: only “Results” should be checked
 - b. Select one file or separate files: pick “One File”
 - c. Enter the export file properties: Export File Name: “_data.xls”
 - d. Choose an appropriate location
2. Open the “WSP QIAgility” workbook
3. On the “Samples” sheet enter the sample names in the order they will be loaded on the QIAgility. Positive and negative amplification controls do not need to be listed. The sample names must be unique and appear exactly as they were on the quantification. The maximum number of samples that can be entered is 62.
4. Enter the “Amp Batch Name” on the “Samples” sheet
5. Enter the “Plate Name” on the “CE Plate” sheet
6. Click the “Choose Folder for Run Files” button on the Samples sheet to save the generated files appropriately
7. Click on the “Import 7500 Results” button on the “Wash. State Patrol” tab in the menu bar
8. The target values on the “Samples” sheet automatically populate to “1” but can be manually updated as appropriate. An amp target that is not possible will automatically be highlighted.

Note: A sample with a quantification value >50 ng/μL will need manually diluted prior to using the QIAgility for normalization. The corresponding quantification value will need updated manually before proceeding to the next step. Likewise, the quantification value for a concentrated sample will need updated manually before proceeding to the next step. Caution should be taken to ensure the volume required by the QIAgility (up to 15 μL) does not exceed the volume in a sample extract.

9. Click the “Create Files” button on the “Wash. State Patrol” tab in the menu bar which will automatically populate the “CE Plate” sheet of the workbook and save the file needed for the QIAgility (amp batch name “_6C_QGility.csv”) and 3500 CE setup file (CE plate name “_3500File.txt”)

QIAgility Normalization and Amplification Setup

1. Mix the appropriate volumes of amplification reagents in a 5 mL tube. Place this tube into position C on the Master Mix Block.

2. Place the appropriate diluents in 5 mL tubes, dilution plate (if needed), and positive and negative amplification controls in the proper positions on the QIAgility Deck.
3. Skipping wells A1 and A2, load samples in EZ1 screw cap tubes into the Sample banks racks in the order designated by the “WSP_QIAgility” Samples sheet. If your samples are in different tubes, be mindful that the instrument is calibrated to the EZ1 screw cap tube, or transfer sample into an EZ1 screw cap elution tube.
4. Place an appropriate amplification plate (or tubes with detached caps) in the appropriate location of the QIAgility Deck.
5. Open the appropriate QProtocols
6. In the Software, select/highlight the rack where your samples are located. Click on the “Import” button. Click on the box to the right of the Import File drop-down menu and navigate to the **amp batch name “_6C_QGility.csv” file** generated above (step 9).
7. Generate the sample banks, making sure the following values are correct:
 “From Row:” = 2
 “To Well:” = A3
 “Limit Sample Count to:” = 64
 “Sample name from column:” = 1
 “Load Conc. from column:” = 2
 Under “Sample Bank Options,” select “Add Filtered Rows to Sample Bank” and “Banks specified in column:” = 3
 Click the “Import” button, then “Finish.”
8. On the QIAgility deck map within the software, double-check reaction steps; depending on the protocol in use this can be done by selecting the destination plate. Delete any pipetting function not in use (grayed out).
9. A pre-run report may be used to confirm that tips, tubes, plates and liquids have been set up correctly. It can be accessed by clicking the icon on the toolbar or the Pre-run Report button at the bottom of the checklist window after clicking the green arrow.
10. Click the green arrow at the top to start. When prompted to Save, create a folder with the batch date. The default name for the file can be accepted. Save the file to the newly created folder.
11. Check the boxes in the checklist menu as the actions are completed.
12. When the run is complete, cap and store the remaining sample extracts as appropriate (the dilution plate may be discarded). Remove the amplification plate/tubes and cover the reaction wells and proceed with the amplification process.

Plexor® HY Quantification Assay Setup

1. Enter sample names into the QIAgility Template page of the QIAgility Plexor Template. This template mirrors the two 4 x 8 well racks of the instrument deck. The sample order will automatically be transferred to the Plate Template sheet matching the 96-well quantification plate setup. Save the QIAgility and the Plate Document sheets as .txt files.
2. The reagent volumes needed are calculated and listed on the Plate Template sheet. Mix the reagents as called for in a 5 ml tube. Place tube into position C on the Mix Block.

3. Place prepared DNA Standards and NTC in their proper positions of the QIAgility Deck.
4. Load samples in EZ1 screw cap tubes into the 4 x 8 well sample racks in the order designated by the QIAgility Template spreadsheet. If your samples are in different tubes, be mindful that the instrument is calibrated to the EZ1 screw cap tubes, or transfer sample into an EZ1 screw cap elution tube.
5. Place a 96 well optical reaction plate in the proper location of the QIAgility Deck.
6. Open the Plexor Quant protocol from the QProtocols folder.
7. In the Software, select/highlight the rack where your samples are located (Position B1 & C1). Click on the “Import” button. Click on the box to the right of the Import File drop-down menu and navigate to the QIAgility .txt file generated above (step 1).
8. Generate a sample bank, making sure the following values are correct:

“From Row.” = 1 (indicates the beginning of the samples to import by skipping header)
 “To Well.” = A1
 “Limit Sample Count = 64”
 “Sample Name = Column 1”
 Uncheck the “Load Conc. From Column” box.
 Under “Sample Bank Options, select “Add Filtered Rows to Sample Bank” and select “QF Samples” under Existing Bank.

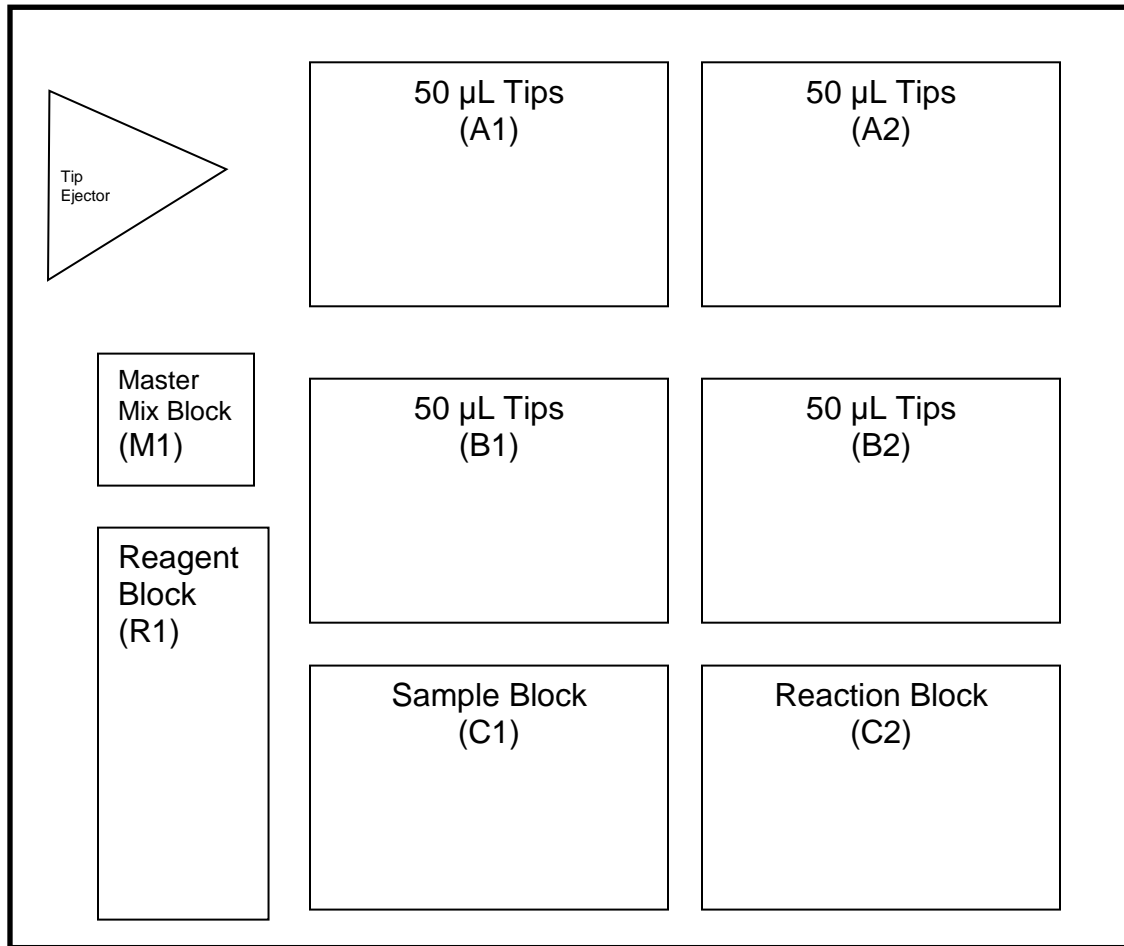
Click the “Import” button, then “Finish.”

In the sample bank window the number following bank “Bank QF Samples” should now correspond to the number of samples in the sample racks.

Note: If samples appear to have imported incorrectly, the sample bank can be edited by selecting the sample bank from the list in the top right window, clicking on “Edit Bank”, then under Sample Selection of the Update Sample Bank window, click on “Bank Wells” to select all the samples and “Delete Selection.” This should be followed by hitting the “Clear” button above the samples list. Then, re-import your samples following the directions in steps 7-8.

9. On the QIAgility deck map within the software, double-check reaction steps; depending on the protocol in use this can be done by selecting the destination plate. The “Pipette 2ul of QF samples...” reaction listed should be active (formerly grayed out) after populating the QF Samples sample bank.
10. A pre-run report may be used to confirm that tips, tubes, plates and liquids have been set up correctly. It can be accessed by clicking the icon on the toolbar or the Pre-run Report button at the bottom of the checklist window after clicking the green arrow.
11. Click the green arrow at the top to start. When prompted to Save, create a folder with the batch date. The default name for the file can be accepted. Save the file to the newly created folder.
12. Check the boxes in the checklist menu as the actions are completed.

CE PLATE SETUP



Procedure

1. Turn on the QIAgility and associated computer. Ensure that the lid of the QIAgility is closed and start the QIAgility software.
2. Open the appropriate plate loading protocol.
3. Adjust the number and location of samples in the sample bank to reflect the number and location of samples in the 96 well amplification plate. Select the Sample Block (robot position C1) then select "Bank Samples" and edit the sample bank by adding or removing samples as necessary.
4. Ensure that the CE does not inject from an empty well by adjusting the number of additional formamide/ILS wells in the second to the last step of the reaction list.
5. Place supplies in the appropriate locations as indicated by the protocol or as follows:
 - a. Place an uncapped 1.5 mL tube of prepared Formamide/ILS (refer to the "Amplification Product Preparation" section of this manual) into the appropriate position of the Master Mix Block. - Ensure that there is an appropriate volume of this reagent for the number of samples to be added to the plate.
 - b. Place an uncapped tube of the appropriate ladder in the appropriate position of the Reagent Block. Ensure that there is an appropriate volume of ladder present in the tube.

- c. Remove the caps from the 96 well amplification plate/tube containing amplified samples and place the plate/tubes into the Sample Block (robot position C1) with well position A1 in the upper left corner.
 - d. Place a new 96 well CE plate into the Reaction Block (robot position C2) with well position A1 in the upper left corner.
 - e. Ensure that there are an appropriate number of 50µL tips present in the robot.
6. Click on the run icon to start the protocol. Save the file
7. A pre-run “Checklist” dialog box will appear. Check the boxes next to the messages that appear in the checklist. All boxes must be checked before the “OK” button will be active allowing the run to proceed. If desired, the “Pre-Run Report” can be viewed by clicking the appropriate button. The number of tips and amounts of other consumables required are listed in the Pre-Run Report.
8. At the completion of the run, cover the CE plate with septa. Refer to the “Amplification Product Preparation” section of this manual to finish preparing the loaded plate.

GUIDELINES FOR EVALUATING DNA PROFILE DATA

Guidelines are provided for evaluating DNA typing profiles obtained by STR analysis using the ~~®-Plus~~, Promega PowerPlex® Fusion 6C and Applied Biosystems Yfiler® PCR Amplification kits, an AB Genetic Analyzer, and GeneMapper® ID-X.

GENEMAPPER® ID-X: DATA ANALYSIS

Work flow analysis for data generated on a Genetic Analyzer processed through the GeneMapper® ID-X program follows.

GENEMAPPER® ID-X: CREATING A PROJECT

1. In the new Project window, select the **Add Samples to Project** icon (or select Add Samples to Project from the Edit menu). Under the Files tab, select the run folder(s) containing the sample files, then click **Add to List** followed by **Add**.
2. In the Project window, select a desired view from the Table Setting drop-down list.
3. Select the **Samples** tab. Assign as appropriate the following parameters: sample type, analysis method, panel, size standard, and custom control. For sample type, reagent blanks and negative amplification controls are designated “Negative Control”, positive amplification controls and custom controls are designated “Positive Control”, ladders are designated “Allelic Ladders”, and all other samples are designated “Sample”. The positive control, custom control or an allelic ladder assigned as a sample can be used as a genotyping control to serve as a QC function as needed.
4. An allelic ladder must be present in each run folder in the project in order to genotype the samples properly. Should the allelic sizing ladder(s) in the run not work, then a ladder from another run from the same rack or sample set from the same Genetic Analyzer can be imported if needed to analyze those samples. A ladder(s) can be re-loaded if necessary and the data imported. This shall be documented in the case file.
5. Once all of the parameters have been assigned, click on the analyze button (green arrow). Complete the fields in the Save Project dialog box. Click OK to save the project.

NOTE: Depending on the Project Options that are set for analysis, the software may not complete the analysis of the project if one or more analysis requirements are not met. This is indicated by a yellow triangle flag (Analysis Requirements Not Met (ARNM)) in the samples. The offending sample(s) are filtered into the Analysis Requirements Summary (ARS) dialog box. Place the cursor over the yellow ARNM flag(s) to display the reason (tooltip) and make the appropriate edit(s) to the sample(s). Repeat step 5.

6. In the navigation pane, expand the run folder. Select the **Raw Data** tab in the content pane then scroll through each of the listed samples in the expanded run folder. Verify the presence of primer peaks in all samples. The raw data may also help evaluate any anomalies, start and stop points for analysis, and the causes of poor size-calling.

GENEMAPPER® ID-X – QUALITY ASSESSMENT

The GMID-X software program analyzes the CE data and allows the analyst to evaluate allelic ladder, control, and sample quality; investigate sample-level process quality values (PQVs) and marker-level

PQVs; review sample plots and edit peak labels; and adjust plot displays to determine the source of artifacts.

The PQV results of the quality assessment are displayed as color-coded flags

- Pass (green square)
- Check (yellow triangle)
- Low Quality (red octagon)

Evaluate the quality flags. It is sometimes necessary to confirm a called artifact, label an artifact, evaluate flagged imbalanced peak heights, review/verify a potentially mixed-source sample, and investigate other low quality flags. Once the data has been evaluated, the sample data can be accepted with the CGQ overridden or the sample data can be rejected and deleted from the project.

Peak Edits

Allows individual peaks to be examined and edited. Left-click to select the peak to be examined and then right-click to open a drop-down menu containing peak edit options

- **Delete Label(s)** – allows the user to delete the label for a particular peak. This peak edit will only be used for preparing CODIS export files.
- **Add Allele Label** – allows the user to add a custom allele label. The custom allele will be added to the marker and will be entered as an allele in the Genotypes table.
- **Rename Artifact Label** – allows the user to customize an artifact label and add it to a predefined list. The custom artifact label is not added to the Genotypes table
- **Peak Raw Data** – allows the user to view the peak morphology in the raw data plot in the Project window.

NOTE: The software automatically labels spikes as such. Any artifact that is labeled by the software or identified by the user as an artifact is not considered an allele by the software and is therefore not listed in the Genotypes table.

Overriding the GQ and CGQ PQV

Once the edit(s) are complete for the marker(s), right-click the GQ flag in the highlighted marker row of the Genotypes table and select **Yes** in the dialog box to override the genotype quality for the marker.

1. The GQ PQV turns to a passing flag (green square).
2. All other PQVs for the marker turn gray and maintain their original shape to indicate the marker was overridden.
3. Once all the PQVs for a sample have been addressed, a dialog box will appear stating that the Genotype Quality (GQ) flag is green for all markers in the sample and ask if the user wants to override the Composite Genotype Quality (CGQ) for the sample. Select **Yes** to override the CGQ.

Overriding the CGQ PQV Only

Alternatively, once the sample has been evaluated for peak height imbalances and all artifact peaks have been re-labeled, right click the yellow triangle or red octagon shape under the CGQ heading. Click Yes in the dialog box to override the Composite Genotype Quality flag for the profile.

Mark Sample for Deletion

Allows the user to mark a sample for deletion and continue to evaluate data. To delete a sample from the project, select the **Mark Sample for Deletion** box at the top right of any dye pane for the sample. Click the **Bring Marked Samples to Top** icon in the Samples plot toolbar. Prior to closing the plot window, verify that the sample(s) marked for deletion is to be deleted. The sample(s) will be deleted from the project when the Samples plot is closed.

GENEMAPPER® ID-X – DATA EVALUATION

All samples regardless of quality assessment flag are examined. Controls and ladders displaying a green passing quality flag may not need to be visually inspected. If a control displays a yellow or red quality flag, it shall be visually examined.

If a load volume and/or injection time other than the validated defaults were employed for the analyzed sample(s), including quality control samples, then those parameters must be documented in the case file.

WEN Internal Size Standard – Fusion 6C

If the sizing quality (SQ) flag is red, evaluate the WEN ILS-500 data to confirm peaks are properly labeled. If there is mislabeling of peaks due to a fast or slow run, create a new “fast” or “slow” version of the analysis method that begins evaluating data sooner or later, as appropriate. If the data indicates a faulty injection, re-inject the sample.

The allelic ladder has PCR products up to approximately 485 bp so the 475 and 500 bp peaks of WEN should be present. If they are not, confirm that there are at least 2 WEN peaks larger than the largest allele in the profile. Allowances will be made for runs that do not include the 500 bp peak if the sample does not have peaks greater than 475 bp. Allowances will also be made for the allelic ladder run. If there are peaks above 475 bp in a sample amplified with the Fusion 6C kit, and no 500 bp WEN peak is present, then the sample must be re-injected. An appropriate allelic ladder with the WEN 500 bp peak must also be used. Ensure that WEN peaks at least meet stochastic threshold for 3500-generated data. Document any modifications to the size standard or analysis method in the case file.

Raw or Unanalyzed Data

The raw or unanalyzed data for each sample should be examined. Typically, the primer peak should rise and fall abruptly and the baseline should be flat and smooth. If the primer peak trails excessively or the baseline is excessively elevated and the genotype data quality is adversely affected, the sample should be re-injected.

Excessive or Saturated Peak Heights

Peak heights should typically not exceed a level where the genotype data quality will be adversely affected. Excessive peak height at the Amelogenin locus is acceptable. In addition, excessive peak height at no more than one locus in single source evidence samples and no more than two loci in reference samples or positive controls is allowable, provided that interpretation of the allele(s) at that locus is unhindered and any resulting artifact(s) (pull up, etc.) does not affect interpretation of other loci. For those samples that do not meet the above-mentioned exceptions, attempts shall be made to resolve excessive peak heights (refer to the “Amplification Product Preparation” section of this manual). A notation will be made in the case file regarding the parameters employed for the analyzed sample.

Allelic Ladder

An appropriate allelic ladder will be included with every set of samples run on the CE unit. Ensure that the allelic ladder displays a green passing flag.

Quality Control Samples

Verification that samples have been extracted and amplified correctly is through assessment of control samples. Passing data does not need to be manually reviewed; non-passing data shall be manually reviewed.

All no template DNA samples (negative control samples and reagent blanks) shall be analyzed with peak detection set at analytical threshold to assist in the detection of contamination.

Positive control samples and custom control samples shall be analyzed with peak detection set at stochastic threshold

- **Reagent Blanks:** No PCR product should be detected in the reagent blank samples. If reproducible peaks are present, the contamination flow chart in the DNA Quality Assurance manual should be consulted. The reagent blank shall be amplified utilizing the same primers, amplification instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA and shall be typed utilizing the same analysis instrument model, injection conditions and most sensitive volume conditions of the extraction set.
- **Confirmatory Samples:** When used, the STR confirmatory sample (SCS: DNA from a case reference sample previously run) shall yield the correct allele designations and be free of contamination.
- **Positive Amplification Control (+C):** Confirm that the alleles in the positive amplification control (+C) are correct and no contamination is present. If the positive control has peaks below stochastic threshold but a second positive control sample (SCS or verified known sample) has normal heights, inaccurate pipetting during +C preparation is indicated and the sample set can be re-amplified or interpretation of the results can be made with caution based on a case-by-case assessment.
- **Negative Amplification Control (-C):** No PCR product should be detected in the negative amplification control (TE buffer/ -C). If the -C has reproducible peaks, the contamination flow chart in the DNA Quality Assurance manual should be consulted and the corresponding samples may need to be re-amplified (if possible). The -C must be loaded and injected at least as long as the corresponding +C.

STR DNA Profile Data

All non-artifact peaks that are sharp, distinct and greater than or equal to analytical threshold will be considered alleles, documented, and considered during profile determination.

The determination of a profile or individual genotype is aided by the use of thresholds established via validation.

Analytical Threshold: 70 RFU (120 RFU for direct amplification samples)

- The analytical threshold (or detection threshold) is the level at which a peak can be reliably differentiated from background fluorescence as either an allele or artifact as determined via validation.
- **Stochastic Threshold: 600 RFU (Semi-Quantitative Binary Approach Only)**
The stochastic threshold is the level at which a sister peak to an allele that meets or exceeds this threshold should be detected above the analytical threshold. Alleles detected between 70 RFU and 599 RFU could have an undetected heterozygous sister allele due to stochastic effects of the PCR amplification process. Allelic dropout, the presence of more than one contributor, and elevated artifacts or background signal shall be considered in the evaluation of the profile data. The stochastic threshold aids in the determination of genotypes within a profile.

- Reference samples and positive controls amplified using standard procedures shall be analyzed with peak detection set at 600 RFU. Reference samples processed by direct amplification may be analyzed with peak detection set at either 120 RFU or 500 RFU.

PCR and Genetic Analyzer Artifacts

If an artifact can be confidently characterized as one of the following anomalies, the sample does not need to be re-injected. Any uncertainty about an anomalous peak should be re-run to ensure quality and accuracy of allele calls.

Artifacts which may interfere with possible alleles shall be considered and documented.

An artifact (spike, shoulder, pull-up or other anomaly) is acceptable provided:

- It meets the criteria outlined below and is appropriately edited by the analyst in the software.
- The peer reviewer of the data agrees that the artifact has been appropriately characterized by the analyst, documented, and is not due to contamination or another problem.

- Stutter**

Stutter is a phenomenon that occurs during PCR due to strand slippage. As a characteristic small peak, one repeat unit less in size than the allele, it is a known biological amplification phenomenon. Established stutter percentages aid in discriminating between stutter and potential extra alleles or some other anomaly (e.g. weak heterozygous or tri-allelic peaks, contamination).

Stutter filters set in GeneMapper® *ID-X* using the values below may be used to filter out potential stutter peaks. In a mixed source sample, stutter peaks may need to be considered. Refer to GeneMapper® Analysis Methods in this manual for the application of stutter filters in sample analysis.

Stutter threshold values (% of alleles) are as follows:

Fusion 6C*			
Locus	n-4 (n-3) % stutter	n-2 % stutter	n+4 % stutter
Amelogenin	--	--	--
D3S1358	13.5	--	1.7
D1S1656	14.3	3.6	2.3
D2S441	9	--	1.8
D10S1248	13	--	1.3
D13S317	10.3	--	2.2
Penta E	7.2	--	1.9
D16S539	12	--	3
D18S51	14.6	--	2.8
D2S1338	13.6	--	2.2
CSF1PO	11.1	--	3.7
Penta D	4.5	--	3.7
TH01	4.8	--	1.5
vWA	14.4	--	2.7
D21S11	12.7	--	2.8
D7S820	9.7	2 *	1.8
D5S818	11	--	2.3
TPOX	5.4	--	1.1
D8S1179	11.8	--	3.4
D12S391	17.4	--	2.7
D19S433	12.1	1.4	2.6

SE33	16.1	6.6	3.3
D22S1045	16.8	--	9
DYS391	9.4	--	2
FGA	12.4	1.2	2.8
DYS576	12.5	--	3.4
DYS570	13	--	2.4

Values from Promega's developmental validation, confirmed through internal validation

* Values from internal validation studies

Peaks in both the minus and plus stutter positions with ratios greater than the above values may indicate the presence of a mixture. Consider heterozygote allele pairing expectations, the number of contributors based on the other loci, and the estimated proportions when evaluating profiles to determine if the elevated or observed stutter could be an allele.

Peaks in the n-8 stutter positions were observed during validation of the Fusion 6C amplification kit; however, they were less common. Refer to internal validation studies for n-8 stutter instances. When determining whether a peak in the n-8 position is stutter or an allele, consider heterozygote allele pairing expectations, the number of contributors based on the other loci, and the estimated proportions.

- DNA-Dependent Artifacts in Fusion 6C

Locus	Artifact Size
Amelogenin	n-1
D1S1656	n-1, n-2
D13S317	n-2
D18S51	n-2
vWA	n-2, elevated baseline in the locus
D7S820	n-2
D5S818	n-2
D19S433	n-2
SE33	n-2
DYS391	n-1
FGA	n-1, n-2

- Spikes**

Spikes have a general appearance of being needle-like and are randomly seen in samples. Spikes typically disappear with re-injection. Commonly, spikes are observed in most or all of the dye colors, and generally at the same base pair location. Occasionally, single color spikes may also be observed. The heights of spikes (in RFU) usually vary between dye colors.

GeneMapper® *ID-X* will automatically flag spikes within the analyzed range. Evaluate the flag and confirm the spike. Spikes may sometimes be flagged as something other than spike. Pull-up can sometimes be labeled as a spike also. Evaluate the artifact and follow the peak edit steps to appropriately flag the artifact.

- Formamide/Dye Blobs**

Raised baseline areas may represent formamide/dye blobs which usually occur at the same base pair location in multiple samples of the run.

GeneMapper® *ID-X* may label a formamide/dye blob that falls within the analyzed range as an allele or with a quality flag. Evaluate the artifact and follow the peak edit steps to appropriately flag the

artifact. If a dye blob falls outside of the marker range but not between markers, GeneMapper® ID-X will not always flag the artifact. In this instance, the artifact can be ignored.

- **Pull-Up**

Pull-up artifacts are peaks that show up at the same base pair size or close (i.e. about a base equivalent on either side of the allele) but in another color. Pull-up usually occurs under peaks with excessive signal or strong alleles. It may also occur between peaks of another color.

GeneMapper® ID-X may label a pull-up that falls within the analyzed range as an allele or with a quality flag. Evaluate the artifact and follow the peak edit steps to appropriately flag the artifact. If a pull-up falls outside of the marker range but not between markers, GeneMapper® ID-X will not always flag the artifact. In this instance, the artifact can be ignored.

- **Shoulders**

Shoulders (-A or +A) flank the sides (one or both) of an allele. GeneMapper® ID-X will not always flag shoulder peaks. If shoulders are present greater than the stochastic threshold and are given an allele designation by GeneMapper® ID-X, the sample should be considered for a re-run at a shorter injection time, run following re-extension, or re-amplified using less template DNA in order to use the data at the locus. If shoulders greater than the stochastic threshold are present in known reference DNA samples and positive controls, then a re-run or re-amp is not necessary, provided that interpretation of the DNA profile is unhindered. If shoulders are at no more than one locus in single source evidence samples then a re-run or re-amp is not necessary, provided that interpretation of the allele(s) at that locus is unhindered and any resulting artifact(s) (pull up, etc.) does not affect interpretation of other loci. The artifact shall be evaluated and appropriately addressed in the software.

Variant Alleles

If a variant (off ladder) allele (any allele peak that does not fit into the current range defined by the GeneMapper® ID-X bins for that locus) or tri-allele pattern is encountered in a DNA sample, the NIST STRbase Web site <http://www.cstl.nist.gov/div831/strbase/> shall be checked for variant allele reports to see if the variant allele or tri-allelic pattern has been previously reported. A printout of this information will be included in the case file.

If a variant allele or a tri-allele pattern has been previously documented at the NIST STRbase Web site, the sample shall be confirmed by a second injection to verify and correctly report the result. Other matching samples from the same contributor qualify as confirmation.

If a variant allele or a tri-allele pattern has not been previously reported at the NIST STRbase Web site, then the sample will need to be reinjected, at a minimum, to confirm the result.

Abnormal sex chromosome variant samples (e.g. XXY) do not need to be re-tested.

A variant allele present in an uninterpretable profile or at RFU levels consistent with the uninterpretable portion of a mixture does not need to be confirmed.

EXPORTING FILES FROM GENEMAPPER® ID-X FOR STRMIX™

1. Choose Table Setting: **STRmix Export**
2. Select the samples that will be utilized in STRmix™ in the **Samples** tab. Multiple reference and evidence samples can be exported at one time into one text file.
3. Choose the **Genotypes** tab.

Notes about this table: it should have “Sample File”, “Marker”, 20 “Allele” columns, and 20 corresponding “Size” and “Height” columns. Ensure the box under “Allele Position” is not checked.

4. Export table by choosing “**Export Table**” from the **File** menu, and save to desired location.

INTERPRETATION OF STR PROFILES - SEMI-QUANTITATIVE BINARY APPROACH

The interpretation of STR profiles takes into account multiple factors. To reduce the risk of potential bias in interpretation, analysts perform the interpretation of unknown samples to determine number of contributors, contributor genotypes, and which loci are suitable to be used for statistical calculations prior to making any comparisons to reference sample data.

Three zones for expected heterozygous peak height ratios (PHR) were established for Fusion 6C. Where at least one allele

- is above 4000 RFU, the PHR is expected to be at or above 60%.
- is between 1300 RFU and 3999 RFU, the PHR is expected to be at or above 40%.
- is between 600 RFU and 1299 RFU, the PHR is expected to be at or above 25%.

Profiles (whether single or multiple contributor) with alleles within and near the stochastic range will be interpreted with caution. The possibility of allelic dropout (not observing a heterozygous sister peak) increases in this range. Loci with peak(s) below the stochastic threshold require special considerations. Data in the stochastic range can be used for inclusions and exclusions if the possibility of allele dropout can be accounted for and can be considered for certain CODIS purposes.

The following interpretations can be made to single and multiple contributor samples:

- **Match (Inclusion)**
Any situation where there are no forensically significant (unexplained genetic) differences between the allele calls obtained from the reference sample and the questioned sample. Statistical analysis must be performed in support of any inclusion that is determined to be relevant in the context of a case.
- **Non-Match (Exclusion)**
If a single allele peak does not match for any locus between the reference sample and the question sample, and in the absence of any scientific explanation of the non-match, then the donor of the reference sample may be excluded as a possible contributor of/to the questioned sample.
- **Not Interpretable, Inconclusive or Limited Interpretation**
Some DNA typing profiles are too low level or are too complex for a variety of reasons (number of contributors, degradation, possibility of dropout, etc.).

A reportable locus is one used for statistical calculations.

Single Contributor Samples

A profile can be considered to be from a single contributor if there are no more than 2 alleles at each autosomal locus and no more than one allele at each Y locus greater than or equal to the analytical threshold as set in GMIDX. Occasionally, three alleles may be detected at any one autosomal locus or

duplication at a Y locus (see “Y-STR Guidelines for Evaluating GeneMapper Data – Consideration of the Occurrence of Duplication” section of this manual) may be observed. If there is no other indication of more than one source of DNA, it is permissible to assume a single source.

If a profile is determined to be from a single contributor and has at least one locus in which the genotype is above stochastic threshold, the profile including alleles above the analytical threshold may be used for inclusionary purposes, statistical assessment, and CODIS uploads (NDIS and SDIS). Y-STR loci may be used for exclusionary purposes and CODIS, but will not be included in the statistical assessment.

Multiple Contributor Samples (Mixed Samples)

If more than two allele peaks greater than or equal to the analytical threshold occur at multiple loci (two or more), then the profile should be considered as having originated from more than one individual. Occasionally, three alleles may be detected at any one locus. If there is no other indication of more than one source for the DNA it is permissible to assume a single source.

When determining the presence of a mixture, the entire DNA profile needs to be evaluated with regard to the following: number of contributors, peak height ratios, degradation, allele masking, allele stacking, etc. For instance, peak height ratios of heterozygous alleles less than the minimum expected could indicate the presence of more than one contributor in the evidence sample and should be considered. This is particularly true for low-level or partial profiles.

Determine the minimum number of potential donors by evaluating the loci with the most alleles detected. To establish the number of contributors, all alleles for that number of contributors must be above stochastic threshold at a minimum of one locus with no alleles observed below stochastic threshold.

Mixture interpretations will generally be limited to two and three person contributor mixtures. Mixtures with four or more contributors are generally un-interpretable; however, a major component can sometimes be present. If a major component is suspected, interpretation of the sample can be done. The argument for the major profile should be documented in the case file. Consultation with and approval by the DNA Technical Leader, Supervisor, or Technical Lead following agreement between analyst and reviewer shall be documented in the case file.

The Y-STR loci may be used to estimate the minimum number of male contributors in a mixture. Y-STR loci may be used for exclusionary purposes and CODIS, but will not be included in the statistical assessment.

Interpretation of Multiple Contributor Samples

Interpretation entails the use of one or more of the following:

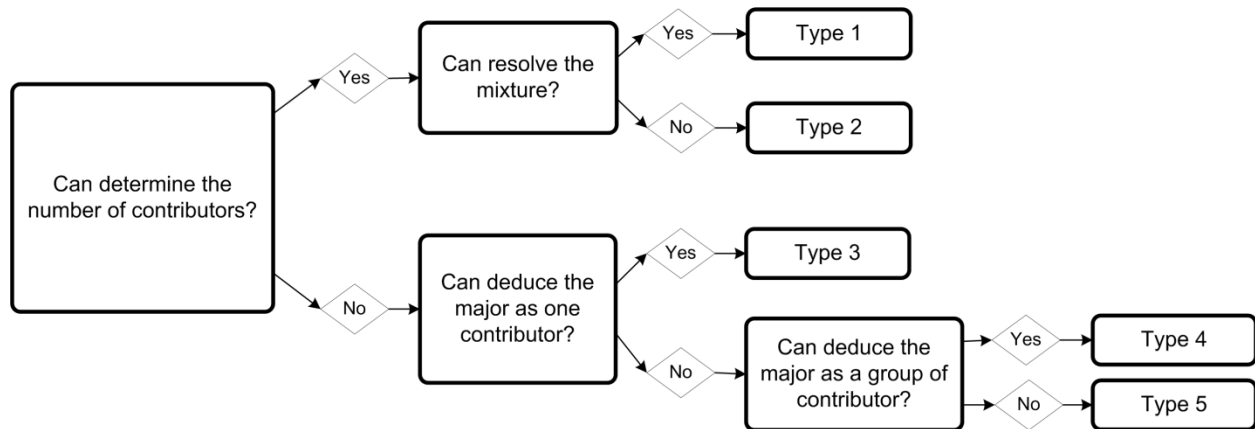
- number of contributors (restricts the number of genotype pairs/combinations),
- presence of known contributors (application of a reference),
- quantitative peak height information (restricts the number of genotype pairs regardless of number of contributors),
- mixture ratio assessments (assists in ruling out some possible genotypes combinations), possibility of drop-out.

Mixture Classification Scheme

If the mixture can be based on an assumed number of contributors, it will be reported out as being 'consistent with' that number, and all further interpretations to include statistical analysis will be conditioned on this assumption (e.g. Type 1 and Type 2 mixtures).

If the number of contributors cannot be reliably determined, then the mixture will be reported out as 'at least' a minimum number based on the assessment. All further interpretations to include statistical analysis will be conditioned on this assumption (e.g. Type 3, 4 or 5 mixtures).

Mixture Classification Scheme



Type 1: interpretation is based on an assumed number of contributors (2 or 3) and data of sufficient quality such that it can be resolved into respective contributors (major/minor). These mixtures can also be conditioned on an assumed known contributor or contributors.

Type 2: interpretation is based on an assumed number of contributors, but cannot be resolved into distinct contributors. This interpretation may be used for both 2 and 3 contributor mixtures.

Type 3: the number of contributors cannot be assumed based on the data, so the interpretation involves deducing a major contributor from the mixture. Generally these mixtures are consistent with a single major contributor with indications of at least 2 low level additional contributors. Interpretation of the minor components is inconclusive. Interpretation of the major contributor is unaffected by the inconclusiveness of the minor components.

Type 4: the number of contributors cannot be assumed based on the data, so interpretation involves deducing a major group of contributors from the mixture. Generally, these present as a shared 2 person major and a low-level contributor(s). If a major group of contributors can be determined, the interpretation (inclusions and exclusions) is limited to the major group and the low-level contributor(s) is inconclusive. If a major group of contributors cannot be determined, the mixture is considered inconclusive and not suitable for any comparison purposes.

Type 5: these mixtures are considered inconclusive and not suitable for any comparison purposes. These mixtures are generally characterized by the following:

- the mixture is partial and of low quantity such that there is not enough data to reliably assume a set number of contributors, and it is also irresolvable such that a major or major group of contributors cannot be deduced.

- the case involves biologically related individuals and the mixture in question cannot be resolved into deduced components.
- the mixture consists of 4 or more individuals that cannot be resolved, making it difficult to reliably include or exclude known individuals.

Mixtures with an Expected DNA Profile

In mixtures where a contributor is known or expected to be present (i.e. intimate samples), subtracting out the known contributor's alleles may allow for determination of the remaining profile. If there is ambiguity in determining the genotype of interest, then a likelihood ratio (LR) calculation is the appropriate method for determining the weight of an inclusion/match.

The following will be considered for the presence of a known contributor(s):

- Assumption of ownership for a known contributor can be made for
 - intimate items (e.g. underwear, body swabs, etc.)
 - other personal items (e.g. clothing, bedding, etc.) as long as there is a reasonable expectation of ownership documented in case file (phone notes, etc.). This assumption will be clearly stated in the report.
- Additional known contributor (e.g. consensual partner) when appropriate. This assumption will be clearly stated in the report.
- A single contributor profile obtained in one fraction of a differential extraction may be applied to the other fraction where carryover/premature lysis is suspected.

Mixtures with Deduced Major/Minor Components

The random match probability (RMP) can be used to determine the weight of a match between a deduced major profile and the matching reference sample.

To report a major profile in a two contributor mixed sample, the total minor component must be 25% or less of the locus total peak sum across the majority of the tested autosomal loci. This is equivalent to a 3:1 proportion or greater of a major contributor.

To report a major profile in a three contributor mixed sample, the total minor component must be 20% or less of the locus total peak sum at the majority of the tested autosomal loci. This is equivalent to a 4:1 proportion or greater of a major contributor.

To declare a male major contributor at the Y-STR markers in a multiple-male contributor mixture (generally two males), the following criteria are used:

- The autosomal profile assessment must coincide with the major contributor coming from a single male contributor,
- The major allele at the Y-STR locus must be greater than or equal to 600 RFU, and the minor allele at the Y-STR locus must be less than 50% of the major allele.
- If only one allele is present and that allele is greater than or equal to 600 RFU, it can be attributed to the major.
- The Y-STR markers are not included in the count of markers in determining a major at the autosomal markers, and the Y-STR markers will be interpreted per locus (no assumption of major can be applied across the Y-STR markers).
- If there is a major female contributor with a minor male contributor and a trace male contributor the above criteria can be applied to assess if there is a sufficient major to minor proportion difference between the two male contributors.

To declare a single major profile and provide an RMP, the major must be unambiguously present. The single major contributor profile in a mixture has a genotype combination that meets or exceeds the expected peak height ratios and has a consistent proportion approximating 3:1 (two person mixture) or

4:1 (three person mixture). If there is ambiguity of the single major profile at a locus, the LR method must be used to determine the weight of inclusion for that profile. Caution must be taken with partial and degraded mixtures.

When a major group of contributors is established in a mixture, a LR will be calculated to determine the weight of inclusion.

- When a type 4 mixture is suspected, the major group shall be approximately equal proportions, consist of at least 95% of the total amplified product (TAP) at each locus, and have consistent proportions within the mixture. The total proportion of the low-level contributor(s) cannot exceed 5% of the TAP at any locus. When these requirements are met, a LR can be calculated for the major contributors and the lower contributor(s) is inconclusive. When the requirements are not met, the profile will be considered inconclusive and not suitable for any comparison purposes.

Calculating a Hypothetical Target Peak Height Lesser Contributor Allele Estimate (LCAE)

All of the alleles of a particular or 'target' contributor in a mixture may not be present or observed above the stochastic threshold at all loci. They may have dropped out or may be masked by the dominant contributor or by stutter peaks. When the target contributor's alleles are not readily apparent, the use of a calculated hypothetical target peak height [lesser contributor allele estimate (LCAE)] can help the analyst in determining what has occurred. This estimate would determine whether the absence of an allele at a single locus can exclude a contributor or requires statistical compensation for dropout as described in STATISTICAL INTERPRETATION OF STR DNA TYPING.

At loci where at least one separate target contributor allele is present, divide the height value of the single allele attributable to the target contributor by the sum of the heights of all the alleles at that locus and multiply by 100. This gives the percent contribution of the target contributor haplotype at an individual locus. Repeat this for all appropriate loci. Once the target contributor haplotype percentages have been calculated for all appropriate loci, calculate the average percent contribution of the target contributor haplotype.

In some cases, the lower allele represented at a locus will obviously not be a single allele from the target contributor. It may represent a homozygous allele (2X peak height) or the sister of a shared major allele. The calculated percent values at these loci do not need to be included in the overall average percent calculation. Where a trace contributor is present in addition to a target contributor, and the lowest allele above the stochastic threshold is attributable to the trace contributor, that allele does not have to be used to calculate the target contributor percentage.

The calculated average percent contribution of the target contributor haplotype is used at appropriate loci to calculate the height of a hypothetical target peak or LCAE. The Y-STR loci cannot be used in calculating the LCAE. Multiply the calculated average percent by the sum of the peak heights at a particular locus to obtain the hypothetical target peak height. At loci where no separate target contributor allele is present, the hypothetical target peak height is used to determine whether it is expected that a target allele should be seen or not. If the estimate is equal to or greater than the stochastic threshold and no allele is observed that can be attributable to the target contributor, either the allele is buried under another contributor allele or is buried under stutter. If the estimate is less than the stochastic threshold, then it is possible that the peak has dropped-out due to stochastic events. Note that the closer to the stochastic threshold the estimate is, the less likely that drop-out has occurred. This can assist in the determination of whether a source can be excluded from a mixture and can be used for determining if a locus is included for statistical assessment.

The calculated average percent contribution of the lower contributor can be applied to the Y-STR loci when that lower contributor is determined to be male. A detected Y-STR allele may be lower than the predicted low contributor allele. The comparison of the total peak heights at a Y-STR locus to the Amelogenin Y peak may be more predictive for drop out than the low contributor allele estimate.

When stutter peaks are present in mixed source samples at RFU levels consistent with a lesser contributor(s), there is a possibility that they are associated with or masking alleles of that contributor. If the lesser contributor(s) needs to be accounted for in the statistical assessment, stutter peaks must be included in the likelihood ratio (LR) calculations unless it can be shown they are not masking alleles of the target contributor.

The determination of whether stutter is included in the statistical calculations is based on the following.

1. **NO** peaks are detected below the stochastic threshold.
The peak height proportion of the target component guides the assessment of the filtered stutter peaks. All peaks equal to or greater than stochastic threshold including filtered stutter peaks will be used in the calculation unless the following exceptions are met.
 - a. If the alleles from the target contributor(s) can be unambiguously determined without considering filtered stutter peaks (i.e. a 2 or 3 person mixture with the expected number of peaks and/or peak height ratios fully support any combined peak heights), stutter does not need to be included in the statistical calculations.
 - b. The target contributor(s) cannot be masked by filtered stutter peaks (determined by the appropriate range(s) of acceptable peak height ratio tolerances). Stutter peaks may be omitted from the statistical calculations.
2. Peaks **ARE** detected below the stochastic threshold.
Evaluate the data in the stochastic range and follow the guidelines below.
 - a. If the only peaks below stochastic threshold are filtered stutter peaks and are determined to not be masking alleles from a lesser contributor, then only the peaks greater than stochastic threshold will be used for the calculation. This may include stutter peaks greater than stochastic threshold (see point 1. above).
 - b. If there is a possibility that the target contributor's alleles have dropped out, then the locus will be considered inconclusive and excluded from any statistical calculation.
 - c. If the target contributor's alleles could be in the stochastic range, determine whether the stutter below stochastic threshold could be masking an allele. Use the following guidelines:
 - If there are already two lesser alleles in a two person mixture, separate from the stutter peak(s), then the stutter peak(s) does not need to be considered in the calculation and the locus may be used.
 - If there is one separate target allele in a two person mixture greater than stochastic threshold or if there are no separate target alleles (low contributor alleles), evaluate the stutter according to the following criteria:
 - If the stutter peak(s) is < 25% of the height of a separate target allele in the 600 RFU to 1299 RFU range, the stutter peak does not need to be considered in the calculation. If the stutter peak(s) is ≥ 25%, the stutter below stochastic threshold shall be included in the LR statistic.
 - If the stutter peak(s) is < 40% of the height of a separate target allele in the 1300 RFU to 3999 RFU range, the stutter peak does not need to be considered in the calculation. If the stutter peak(s) is ≥ 40%, the stutter below stochastic threshold shall be included in the LR statistic.

STATISTICAL APPROACHES

Statistical Approaches

Type 1: RMP is used for deduced/distinct contributors (i.e. single contributor or unambiguous major). If major genotype is ambiguous, LR is used for inclusions to the mixture.

Type 2: LR is used for inclusions.

Type 3: RMP is used for the major contributor; the minor component is generally inconclusive.

Type 4: LR is used for the group of major contributors; the minor component is generally inconclusive.

Calculating the Random Match Probability

For single contributor samples and deduced major profiles from Type 1 or Type 3 mixtures, the Forensic Single Sample Mode may be selected to calculate a random match probability.

For a single contributor or deduced major profile in which only one allele is present and it is below stochastic threshold, then the NRC '96 alternative homozygous formula B option ($2p-p^2$) can be used to calculate the RMP for that profile. To use the $2p-p^2$ option, check the NRC box for that locus in the Target Profile screen and ensure the Configuration screen has the $2p-p^2$ option selected.

The use of the alternate homozygous formula B will be denoted on the statistics report by a "P" next to the frequency data for the selected locus.

Likelihood Ratio

The LR calculation may be used to express the weight of an inclusion in a Type 1, Type 2, Type 3, and Type 4 mixed DNA profile. The Type 4 mixed DNA profile assumes there are 2 major contributors.

When all the STR alleles of a mixture profile are sufficiently above stutter levels and stochastic effects are not an issue, the simple unrestricted combinatorial LR approach should be used to consider all possible sets of genotypes.

When the genotype of interest is a minor component, de-convolving the mixture is required to determine if the results are suitable for inclusionary purposes. The possibility of stutter masking or stochastic effects at each locus must be assessed as to whether the genotypes of all contributors are sufficiently represented to include that locus in the calculations. Consequently, all the alleles in the genotype of interest must be present at each locus that was determined to be suitable for the LR calculation. If not, comparison to that reference is inconclusive for the mixture.

When the lesser contributor's alleles are not clearly present, an assessment can be made of whether the alleles are shared/masked or not detected (for example, a single or double allele locus) by a calculation of the hypothetical peak height of the potentially masked lesser contributor (LCAE). Data below stochastic threshold may be used for inclusionary purposes once the appropriate assessments regarding number of contributors, allele masking, drop out, etc. have been conducted.

In loci with no alleles detected below the stochastic threshold, the calculated value must be greater than or equal to the stochastic threshold to ensure that the peaks would have been detected (i.e. above analytical threshold if present). A calculated value lower than stochastic threshold confirms that the lesser contributor's alleles may not be present above analytical threshold and that this locus cannot be used for the LR calculation.

Calculating the Likelihood Ratio (LR)

1. Select Forensic Mixture from the Popstats Calculations menu.

2. For each locus, enter all of the alleles that are to be used for the calculation in the first column, labeled Target Profile – see Accounting for Stutter section text. The appropriate loci and alleles to be included should be determined following the above guidelines (see LR section text). Stutter peaks (i.e. within expected stutter filter percentages) included for statistical assessment remain in the Target Profile column and are not entered into the other columns.
3. The second column, H1, relates to the first hypothesis to be compared (inclusion hypothesis). Enter all of the alleles from the first column that are not accounted for by the first hypothesis (i.e. those foreign to the assumed contributors under this hypothesis). The number of unknowns for H1 should be set as appropriate for the number of unknown contributors in the mixture according to the first hypothesis.

Note: All alleles entered in the first column will be appropriately considered in the calculation even if they are not entered into the second column. Stutter peaks (i.e. within expected stutter filter percentages) included for statistical assessment remain in the Target Profile column (column 1).

- a. For example, if the first hypothesis is that the victim and the suspect are the contributors to a two person mixture, only those alleles in the mixture that are foreign to both the victim and the suspect should be entered into this column; usually this will mean no alleles are entered, and the # of unknowns for H1 should be set to 0.
 - b. For example, if the first hypothesis is that the suspect and an unidentified individual are the contributors to a two person mixture, only those alleles in the mixture that **must** belong to the unidentified individual (i.e., foreign to the suspect) should be entered into this column and the # of unknowns for H1 should be set to 1.
 - c. For example, if the first hypothesis is that the victim, the suspect and one unidentified individual are the contributors to a three person mixture (and it has been determined that the most likely number of contributors is three individuals), the alleles that **must** belong to the unidentified individual (i.e., foreign to the victim and the suspect), will be entered into the second column and the # of unknowns for H1 should be set to 1.
4. The third column relates to the second hypothesis, H2, to be considered (exclusion hypothesis). Enter all of the alleles not accounted for by the second hypothesis (i.e. those foreign to the assumed contributors under this hypothesis). These are the alleles being attributed to the unknown contributor(s). The drop down menu next to the # of unknowns for H2 should be set as appropriate, usually one more than the value set for the second column (H1).

Note: All alleles entered in the first column will be appropriately considered in the calculation even if they are not entered into the third column. Stutter peaks (i.e. within expected stutter filter percentages) included for statistical assessment remain in the Target Profile column (column 1).

- a. For example, if the first hypothesis is that the victim and the suspect are the contributors to a two person mixture, the second hypothesis would usually be that the victim and an unidentified individual are the contributors. Therefore, the alleles that are foreign to the victim should be entered into the third column, and the # of unknowns for H2 should be 1. These are the alleles being attributed to the unknown contributor.
- b. For example, if the first hypothesis is that the suspect and an unidentified individual are the contributors to a two person mixture, the second hypothesis should be that two unidentified individuals are the contributors. Therefore, all the alleles in the mixture that **must** belong to the unidentified contributors should be entered into the third column, and the # of unknowns for H2 should be 2. These are the alleles being attributed to the unknown contributors.

- c. For example, if the first hypothesis is that the victim, the suspect and one unidentified individual are the contributors to a three person mixture (and it has been determined that the most likely number of contributors is three individuals), the second hypothesis would usually be that the victim and two unidentified individuals are the contributors. Therefore, the alleles that are foreign to the victim should be entered into the third column, and the # of unknowns for H2 should be 2. These are the alleles being attributed to the unknown contributors.
5. Select Calculate.
6. Select Print from the File menu and print the LR for Mixtures Report.

Interpretation of STR Profiles from legacy protocols

Occasionally, a DNA typing result that was developed using a protocol that is no longer in use may need to be further evaluated for comparison to newly submitted samples or to assess a candidate match. For Profiler Plus® and COfiler®, the analyst will refer to Revision 12 of the WSP Casework STR Analysis Procedures to find information and interpretation guidelines for these kits. For autosomal data generated using AmpFtSTR Identifiler® Plus amplification kit, the analyst will refer to Revision 28 of the WSP Casework STR Analysis Procedures to find analysis settings and interpretation guidelines. The SOP revision number will be recorded in the case notes.

For autosomal data generated on an Applied Biosystems 3130 Genetic Analyzer, the analyst will refer to Revision 23 of the WSP Casework STR Analysis Procedures to find analysis settings and interpretation guidelines. For Y-STR data generated on an Applied Biosystems 3130 Genetic Analyzer, the analyst will refer to Revision 22 of the WSP Casework STR Analysis Procedures to find analysis setting and interpretation guidelines. Since the 3130 and 3500 are considered the same platform a requalification is not necessary.

Other tasks allowed without documented requalification include:

- 1) Evaluating a candidate match within CODIS from a high stringency search that just involves a comparison between two single source CODIS entries that match at high stringency.
- 2) Comparing a casework (forensic) DNA profile interpretation that previously had the genotypes of the possible contributors documented in the mixture interpretation worksheets compared to the results of moderate stringency search candidate matches.

Tasks that require documented requalification include:

- 1) Moderate stringency matches between a candidate and target DNA profile where: (1) one or both of the DNA profile(s) originate from legacy data; and (2) the match involves comparisons of the original image(s) or electropherogram(s) to assess the match; are considered reinterpretation.
- 2) Assessing/evaluating allele calls, genotype calls (to include potential allelic drop-out), a change in the assumptions used, or removing alleles (or entire loci) from statistical estimates from legacy amplification test kit data, are all considered reinterpretation.

An analyst remains qualified for reinterpretation of data for 2 years after the last proficiency test of a legacy kit (or the last requalification, see below). If two or more years have passed then the analyst must go through the following requalification process.

To requalify an analyst who has been previously qualified in the legacy kit(s) complete the following training plan: 1) review the pertinent validation data (internal lab and system write-ups) and 2) review the most recent and relevant standard operating procedures (posted on SharePoint). Once the analyst has completed this training plan, prepare an IOC stating that the training plan for the legacy amplification kit(s)

has been completed and reference this section of the manual. Send the IOC up through the appropriate chain of command for approvals by the Supervisor, Lab Manager, DNA Technical Leader and Division Manager.

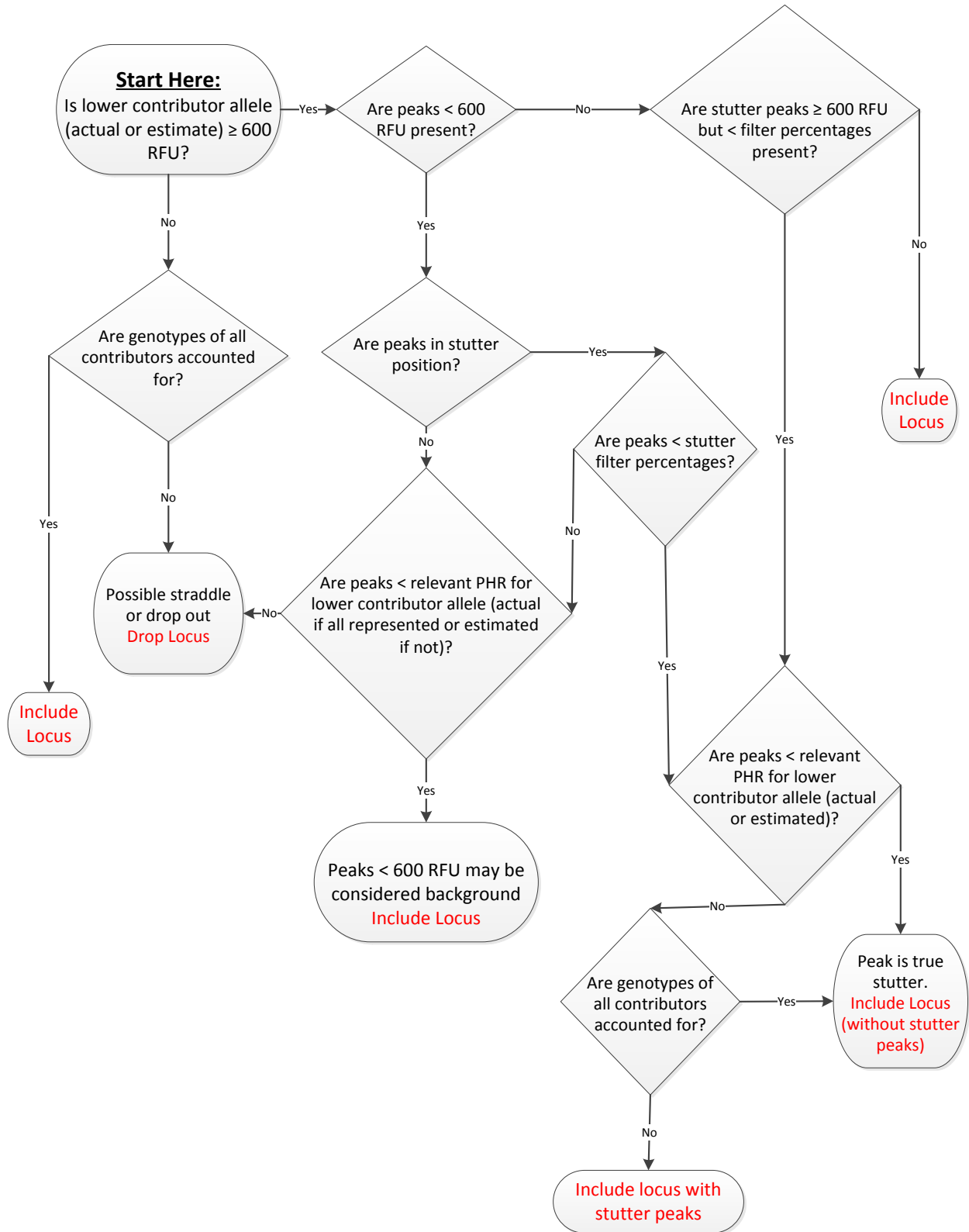
To qualify an analyst in a legacy amplification kit(s) that is proficiency tested in the current amplification kit(s) but was never previously qualified in the legacy amplification kit(s) the following procedure is followed. Prepare a training plan for DNA Technical Leader approval which includes the review of the validation data and standard operating procedures of the legacy kit(s), training by a previously qualified analyst, and an interpretation competency test. Once the analyst has reviewed the appropriate materials, passed the qualified analyst training and passed a competency test, prepare an IOC stating that you have completed the training plan for the legacy amplification kit(s) with the training plan included as an attachment. Send the IOC up through the appropriate chain of command for approvals by the Supervisor, Lab Manager, DNA Technical Leader and Division Manager.

Technical reviewers of reinterpretations of the legacy data are held to the same training requirements as the analyst reinterpreting the data.

3500 GENERATED DATA

Likelihood Ratio (LR) Flow Chart (for Popstats)

- Analysis for stutter is not required if one can account for all alleles of the lower contributor (e.g. 4 peaks in a two person mix)
- A locus can be used for statistical purposes if one can account for the genotypes of the assumed number of contributors



GENEMAPPER® ID-X PROFILE COMPARISON TOOL

The Profile Comparison tool is used to perform quality control of sample results in a project. Only samples and controls with flags of passing (green square) or check (yellow triangle) are included in comparisons. These three functions are performed for all samples in a project:

- Groups samples with 100% concordant profiles (single-source and mixed-source groups)
- Compares samples in the project against all other samples in the project
- Compares samples in the project against lab reference, custom control, and QC sample profiles stored in the Profile Manager.

Terms:

- **Reference Profile** – The profile against which another profile is compared to determine the % Match. May be single source or mixed source (a mixed source sample will only be used to compare to another mixed source sample). The profile will be indicated in bold lettering in the Profile Comparison tool window.
- **Comparison Profile** – The profile compared to the reference profile to determine the % Match. The profile will be indicated in blue lettering in the Profile Comparison tool window.
- **% Match** – Calculated using the following formula:

$$\frac{(\# \text{ reference profile alleles found in comparison profile} / \text{total} \# \text{ reference profile alleles}) \times 100}{100}$$
- **Single-source groups** – single source samples which demonstrate 100% concordance (all markers and all alleles match)
- **Mixed-source groups** – mixed source samples which demonstrate 100% concordance
- **Individual Samples** – Samples that contain unique profiles within the project
- **Lab Reference and Custom Control Profile** – profiles imported and stored in the Profile Manager.

Perform Sample Comparison and View Results

This tool may be used to determine whether any of the sample profiles in the project are potential contributors to another sample profile in the project.

1. In the Project window, select **Tools, Profile Comparison**. The Profile Comparison tool opens to the Sample Concordance tab. To view the samples in the listed group(s), click the '+' to expand the Samples view.
2. Select the appropriate tab within the Profile Comparison tool.
 - a. **Sample Comparison** tab to compare samples to each other.
 - b. **Lab Reference Comparison** tab to compare samples within the project to lab reference profiles stored in the GeneMapper® ID-X database.
 - c. **Control/QC Comparison** tab to perform a blind QC check by comparing the profiles for the custom control and QC samples present in the project to the custom control profiles stored in the Profile Manager.

For each of the tabs listed above, maintain the Percent Match Threshold at 80. To make comparisons, click the **Compare Profiles** button. Review the results.

INTERPRETATION OF STR PROFILES – PROBABILISTIC GENOTYPING USING STRMIX™

STRmix™ is a software program that applies a fully continuous probabilistic genotyping approach to DNA profile interpretation. It standardizes the analysis of profiles in the laboratory by using estimates of variance of results derived from validation data. STRmix™ can be used to analyze samples with or without reference samples. A key component of the software involves Markov chain Monte Carlo (MCMC) methods which comprise a class of algorithms for sampling from a probability distribution.

ANALYZING DATA IN GENEMAPPERID-X FOR STRMIX™

1. To assist the analyst in determining which alleles may possibly be attributed to stutter while the analyst is attempting to determine the number of contributors to a profile, data will initially be analyzed in GMID-X using the following:
 - Analytical threshold (70rfu) for evidence samples and blanks
 - Stochastic threshold (600rfu) for reference samples and positive controls
 - Marker specific stutter filters enabled

This GMID-X project may be retained in the case file.

2. Reference samples for STRmix analysis will be analyzed with the marker-specific stutter filters on.
3. Reanalyze the evidence profiles for STRmix analysis using the created STRmix analysis method (Fusion6C_70rfu_STRMix). Ladders associated with this analysis can be analyzed with the same STRmix analysis method or one with a 600rfu threshold (Fusion6C 600rfu STRMix). These analysis method filters n-2 stutters but retains the standard stutters (N+4/-4). This analyzed data shall be retained in a GMID-X project.
4. Export this data to STRmix.
 - Select the Genotypes tab
 - Select the **Export to STRmix** from the Table Settings drop-down menu.
In the Table Settings Editor, select "Sample Name", "Marker", "Allele", "Size", and "Height" for the column settings. Enter '20' for number of alleles to show. Ensure the box under "Allele Position" is not checked.
 - From the File menu, select "**Export Table**" and save to desired location.
5. A GMID-X project(s) containing the final analyzed files for all samples will be retained in the case file.

Notes:

- STRmix cannot model non-standard stutters (e.g. n+/-2, n-8). The presence of alleles in these positions (which may be stutter peaks or alleles from a true contributor) may cause STRmix to fail due to the number of contributors (NOC) the analyst assigned to the profile.
- Input data derived from an electropherogram (epg) must be formatted in a specific way for analysis. Sample names cannot contain commas ",". Non numeric values such as 'OL' are also not permitted within the STRmix™ input files. Therefore, variant alleles must be assigned the appropriate numeric value (e.g. 33.1) prior to being included in the STRmix™ input file. If the numeric value of a variant allele cannot be confidently assigned (e.g. ">" or "<" 12) the locus at which the variant allele was observed will be ignored during STRmix™ analysis.
- Additionally, STRmix™ cannot accommodate somatic mutations or trisomy. If a profile has a tri-allelic pattern, the locus at which the tri-allelic pattern was observed will be ignored during STRmix™ analysis. If the presence of a somatic mutation is suspected, consult with the DNA Technical Leader.

DETERMINING NUMBER OF CONTRIBUTORS (NOC)

STRmix™ requires an input of the estimated number of DNA contributors in a sample on which to condition its analysis. Assumptions as to the number of contributors shall be based on the most reasonable interpretation of the data. Multiple STRmix™ analyses under different number of contributor assumptions may be necessary in some circumstances (seek Supervisor or Tech Lead approval).

An estimate of the NOC is based on the locus that exhibits the greatest number of allelic peaks. Additional information such as expected peak height ratios at other loci may assist the analyst in determining the minimum NOC.

The following steps will be used by the analyst when estimating the NOC:

1. The profile should be reviewed as a whole, assessing the level of degradation, presence of low level peaks, noisy or clean baseline, and general quality of the profile.
2. Likely stutter peaks (both forward and back) should be considered with reference to the internally validated per allele stutter ratio expectations.
3. The locus with the highest number of unambiguous allele peaks should be identified. The number of alleles divided by 2 (or the number of alleles plus one, if an odd number of alleles are detected) shall be used to determine the initial postulated NOC.
4. Peak height imbalances shall be reviewed at the most informative locus/loci (greatest number of alleles). Consideration of any imbalances, along with the possibility of allele sharing or 'stacking', may indicate the likely presence of an additional contributor above that indicated by allele count alone. An attempt may be made to visually "pair" alleles and assign them as contributors.
5. Possible contributor proportions across the entire profile should be considered; if one or more contributors at an informative locus was either at a trace level or was a clear major, this pattern should be checked to ensure it is represented at other loci. \
6. The general pattern of contributors (number and proportion) should be applied to all loci in the profile to determine if the NOC assigned to the profile appears reliable or if the addition or subtraction of one contributor may be more appropriate. Genetic variants such as trisomy should also be considered at loci where the general pattern does not hold across the profile.

Note: STRmix cannot model non-standard stutters (e.g. $n+/-2$, $n-8$). The presence of alleles in these positions (which may be stutter peaks or alleles from a true contributor) may cause STRmix to fail due to the NOC the analyst assigned to the profile.

The presence of a single allele or few low level alleles above the analytical threshold may infer a larger NOC. In this instance, peaks observed below the analytical threshold not in known artifact positions may be used to support this inference.

The risk of false inclusions increases when additional contributors (more than the true NOC) are assumed to be present in the mixture, while the risk of false exclusions increases when fewer contributors are assumed. Therefore, it is usually more appropriate to bias estimates of NOC towards fewer contributors. Note that incorrect assignment of NOC typically only affects the likelihood ratio and genotype assignments for low level contributors.

If the NOC is ambiguous, the STRmix™ analysis may be run with at least the two most likely NOC and the lowest likelihood ratio (LR) shall be reported. Documentation of both analyses shall be retained in the case file. Performing multiple deconvolutions of one sample with different contributor numbers requires a sign off from a supervisor, Technical Lead, or Technical Leader. If the resulting LRs from the analyses straddle exclusion/uninformative ranges, a deconvolution with 5,000,000 post burn-in accepts per chain with the lower contributor number shall be performed and used for reporting.

Mixtures with no more than 8 alleles at any locus shall be deconvoluted with 4 assumed contributors based on the analyst's evaluation of all the data (a 5 contributor setting can be tested as a diagnostic step to assess the robustness of the 4 contributor estimation). If more than 8 alleles are observed at one or more loci, the profile will be reported as uninterpretable unless the DNA Technical Leader approves the use of STRmix™ to deconvolute the profile (e.g. a sample with a single large unambiguous contributor and indeterminate minor/trace contributors).

Profiles obtained from differential extractions may be manually assessed to determine if one fraction is non-probative (e.g. non-sperm fraction from victim's vaginal swabs matches victim or is primarily from victim with carryover from sperm fraction). These non-probative fractions shall be documented on sample or mixture analysis forms, as appropriate, and do not need to be analyzed using STRmix™.

An analyst may perform manual comparisons for simple exclusions to robust single source profiles or 2-person mixtures. Alternatively, STRmix™ may be used for all comparisons. **REFERENCE SAMPLES AMPLIFIED WITH DIFFERENT AUTOSOMAL PROFILING KITS**

STRmix™ allows the user to calculate a likelihood ratio (LR) when the evidence sample and reference samples are analyzed in different autosomal profiling kits. STRmix will only provide LRs for those loci in common between the two kits. For example if you had an evidence sample analyzed with Fusion 6C and a reference sample analyzed with a legacy kit, STRmix would be able to provide an LR using the loci the kits have in common.

If a STRmix™ deconvolution of a Fusion 6C evidence sample requires an assumed person, whose reference was typed in a legacy kit, it is preferred that the reference sample be re-amplified with Fusion 6C to be used in the deconvolution. If re-amplification is not possible, the deconvolution can be performed without the assumption. An additional option would be to perform the deconvolution with the assumed person using the "Ignore Locus" function for all loci that the evidence sample and reference sample do not have in common. Analysts should be aware that this option will lower the discrimination power of the analysis.

Note: For all reference sample data to be used with STRmix, the full genotype at each locus used must be present for comparisons. If a single allele is entered in a STRmix reference input file, STRmix automatically considers that entry as a homozygote (eg. 11 = 11,11 not 11, Any), therefore partial reference sample loci shall be ignored or not entered.

MANUAL CREATION OF FILES FOR STRMIX™ INPUT

This will be necessary when a reference sample cannot be exported from GeneMapperID-X because it was created by another laboratory, or it was not typed with the Fusion 6C amplification kit. It will also be necessary when conditioning on an unknown profile developed from a mixture for the purposes of deducing another unknown profile for CODIS entry (see Deducing Profiles for CODIS Entry section).

1. A STRmix™ Ref Input File Excel workbook has been created to assist in the creation of the Fusion 6C or Identifiler Plus reference sample STRmix input files. Open the STRmix™ Ref Input File and select the bottom tab corresponding to the amplification kit used to generate the reference profile.
2. Input the sample name in all rows of the "Sample Name" column.
3. Input the profile in the Allele columns – homozygotes need only be entered once in the "Allele 1" column.
4. Select "Save As" from the File menu. Select a suitable save location, enter a File name, and select "Text (Tab delimited)" from the Save as type drop down menu.
5. Click "Save." This file can then be used in the Add Reference Profile Data area of the Add Profile Data window (see Figure 7 and Step 6 of the Single Sample Analysis procedure).

STRMIX™ REPORT CONFIGURATION

1. Select “Settings” from the STRmix™ main menu. Enter the administrator password.
2. Click the “Configure Reports Defaults” button.
3. Ensure settings are as displayed in Figure 12.

Configure and Run Report

General Settings

Lab logo (optional) Select File

Page Size Letter

Auto-Open Report ☒

STRmix Calculation Type: Analysis/LR From Previous

Choose Reports to Run

☒ Summary Report
☐ CODIS Report

Summary Report Settings

Included Components Drag and drop rows to reorder

Component	Include?	New page?
Contributor Summary	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Variance Graphs	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Run Information	<input checked="" type="checkbox"/>	<input type="checkbox"/>
LR Summary	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Component Interpretation	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Evidence Files	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Reference Files	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Stutter Files	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Settings	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Locus Efficiencies	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Genotype Probability Distribution	<input checked="" type="checkbox"/>	<input type="checkbox"/>

LR Summary Settings

Show Unified LR? ☐

Show Stratified... ☐

Cancel Save

STRmix V2.5.11 - User: lhea225

Figure 12

STRMIX™ ANALYSIS

Start STRmix™ by double-clicking the STRmix™ icon on the desktop or select STRmix™ from Start > STRmix™. The STRmix™ main menu will display. Each of the functions within STRmix™ can be accessed from the Startup screen. To exit the software click the Exit button or the top right-hand X button.

Peak heights should typically not exceed a level where the genotype data quality will be adversely affected. Excessive peak height at the Amelogenin locus is acceptable. In addition, excessive peak height at no more than two loci in evidence samples, reference samples or positive controls is allowable, provided that interpretation of the allele(s) at that locus is unhindered and any resulting artifact(s) (pull up, etc.) does not affect interpretation of other loci. Extra care should be taken to ensure the deconvolution results are intuitively correct and have not been adversely affected by the saturated data.

No references will be compared during the initial deconvolution of a mixture unless the donor of the reference can be reasonably assumed. If the analyst is uncertain if a reasonably assumable individual is included as a contributor to an evidence profile, they may deconvolute the mixture without the assumed

contributor and then the assumable individual should be compared to the deconvolution by calculating a likelihood ratio. If the result is less than one, the original deconvolution should be used as the final deconvolution. If the result is greater than one, the deconvolution should be re-run conditioned on the assumable individual. This deconvolution shall be considered the final deconvolution and printed in the case file. The previous deconvolution shall be included in the electronic data but does not need to be printed. Deconvolution results should be carefully examined for unintuitive combinations when the likelihood ratio for the assumed contributor is close to one.

Sometimes the complexity of a profile to be analyzed will be beyond the available computing capacity of the computer running the STRmix™ software. This will either cause STRmix™ to fail during analysis and/or present an out of memory error. Alternatively, STRmix™ may run so slowly that an analysis could take weeks to reach completion. If an analysis does not complete due to an out of memory error, or is taking a very long time to complete, the analysis will be re-run in low memory mode (see Single Sample Analysis step 3 below). If in low memory mode an analysis is still not able to run to completion it will be reported with the following statement:

“An attempt was made to analyze this profile with the STRmix™ forensic software. The analysis requirements exceeded available computing capacity; therefore this profile is unsuitable for comparisons.”

SINGLE SAMPLE ANALYSIS

1. Select “Start Analysis” to open the “Configure Analysis” window (see Figure 1). This initiates a three-step process for the interpretation of single source and mixed DNA profiles.

Figure 1.

2. Fill in the Case Number, Sample ID, any case notes (optional), and the number of contributors for the analysis. The number of MCMC accepts and burn-in accepts should only be changed if longer analyses are required to better resolve complex mixtures.

3. Complex mixture analysis may slow your computer down. If this is a concern, the low memory mode may be used. Low memory mode can be selected by clicking the “Run Settings” button in the Configure Analysis window (see Figure 2). The Run Settings window is also where the seed can be set if needed for performance check purposes. All other settings should not be changed in this window. Click the “Save” button to return to the Configure Analysis Window.

Figure 2.

4. Click the “Confirm” button in the Configure Analysis window to open the Add Profile Data window (see Figure 3). Select the DNA kit used “WSP_Fusion6C_3500” from the drop down menu (you should only need to do this the first time the software is used). If loci need to be excluded from the analysis, click the “Kit Setting” button in this window to open the Kit Setting window (see Figure 4). Values in this window should be as shown in Figure 4 and should not be altered. Click the “Ignore Loci” button to remove loci from the analysis. DYS391, DYS576, and DYS570 should be the default selection (see Figure 5). Other loci may be selected in the left column and moved to the right column for exclusion from analysis using the “>” button. Click the “Save” button in the Ignore Loci and Kit Setting windows to return to the Add Profile Data window.

STRmix - Add Profile Data

Case Details: 216-850 - #1A Sheet Open Results Direct...

Kits in Analysis

WSP_Fusion6C_3500

DNA kit used: WSP_Fusion6C_3500 Kit Settings

Add Evidence Profile Data:

Add Profile Remove Profile

Add Kit Remove Kit

☐ Fix degradation

Add Reference Profile Data

Add Reference Remove Reference Edit Reference Change Hd

Contributor to:

Hp Hd

Cancel Back Confirm settings

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Figure 3

STRmix - Kit Settings for WSP_Fusion6C_3500

Kit Settings for WSP_Fusion6C_3500

Variance		Thresholds	
5.174,1.759	Allelic Variance	70,70,70,70,70,70,70,70,70,70,70,70,70,70	Detection threshold <input type="button" value="Edit"/>
1.852,8.182	Stutter Variance	30000	Saturation
0.01	Locus Amp Variance	0.3	Stutter max
0.5	Min Variance Factor	0.15	Forward stutter max
		<input type="button" value="Ignore Loci"/>	
Degradation			
0.01	Degradation max		
-1.0	Degradation starts at (bp)		
Drop-in			
120	Drop-in cap		
0.0029	Drop-in frequency		
0.0,0.0	Drop-in gamma parameters		

STRmix V2.5.11 - User: lhea225

Figure 4

Ignored Loci

Ignore Loci

Include:		Exclude:
D3S1358	<input <input="" style="display: inline-block; width: 20px; height: 20px;" type="button" value=" ≡ "/>	DYS391
D1S1656		DYS576
D2S441		DYS570
D10S1248		
D13S317		
Penta E		
D16S539		
D18S51		
D2S1338		
CSF1PO		
Penta D		
TH01		
vWA		
D21S11		
D7S820		
D5S818		

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Figure 5

4. The evidence profile to be analyzed can be selected either by dragging the .txt file exported from GeneMapperID-X into the “Add Evidence Profile Data” box and selecting the correct sample from the pop-up window, or by selecting the “Add Profile” button and selecting the correct file and sample under the “Import from plate text file” header (see Figure 6).

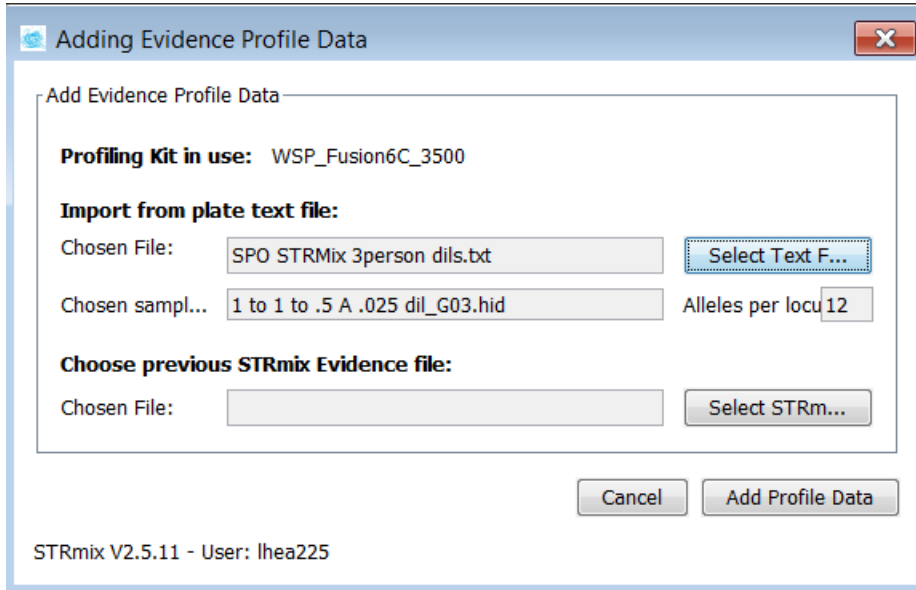


Figure 6

5. If an individual can be reasonably assumed, the deconvolution shall be conditioned on the presence of that individual (e.g. the donor of an intimate sample), and their reference sample shall be included in the “Add Reference Profile Data” area of the Add Profile Data window (see Figure 3) (for exceptions, see additional information under STRmix Analysis above). The reference profile to be used for conditioning can be selected either by dragging the .txt file exported from GMID-X into the “Add Reference Profile Data” box and selecting the correct sample from the pop-up window, or by selecting the “Add Reference” button, followed by the “Add Profile” button in the follow window, and selecting the correct file and sample under the “Import from plate text file” header. Click the “Add Profile Data” button, followed by the “Save Reference” button to return to the Add Profile Data window.
6. To condition on the chosen reference sample, there must be a “X” in both the Hp and Hd columns. Click on the reference sample and then click the “Change Hd” button to add the X to the Hd column (see Figure 7).

STRmix - Add Profile Data

Case Details: 216-850 - #1A Sheet Open Results Direct...

Kits in Analysis

WSP_Fusion6C_3500

DNA kit used: WSP_Fusion6C_3500 Kit Settings

Add Evidence Profile Data:

Add Profile Remove Profile

1 to 1 to .5 A .025 dil_G03.hid_EV.csv

Add Kit Remove Kit

☐ Fix degradation

Add Reference Profile Data

Add Reference Remove Reference Edit Reference Change Hd

Exercise 18 ref_REF.csv

Contributor to:

	Hp	Hd
Exercise 18 ref_REF.csv	X	X

Cancel Back Confirm settings

STRmix V2.5.11 - User: lhea225

Figure 7

- Click the “Confirm Settings” button to move to the Population Setting window. Most areas in this window should be greyed out (see Figure 8) and no setting adjustments should be needed. Click the “Start” button to start the analysis.

STRmix - Population Settings

Case Details: 216-850 - #1A Sheet Open Results Direct...

Population Settings

▼ Add Population Remove Population

Population	Proportion	FST	Allele Freq File

Factor N!

☒ Factor of N! LR

Sampling Variation

☒ Calculate HPD ☒ Include MCMC uncertainty

HPD iterations: 1000 Quantile: 99 Sides: 1

Save as Default Populations Cancel Back Start Start & Search

STRmix V2.5.11 - User: lhea225

Figure 8

- The results files will be located in the STRmix™ Results folder (accessible from the Start Menu in the “STRmix™” folder) and will be named in the following manner: Case Number_Sample ID_yyyy-mm-dd-hh-mm-ss.

BATCH SAMPLE ANALYSIS

- Multiple samples may also be added to STRmix™ to be processed sequentially by selecting the “Batch Mode” button from the Startup screen to access the Batch Mode Progress window. Click the “Add to Batch” button to add the first sample you would like to analyze (see Figure 9).

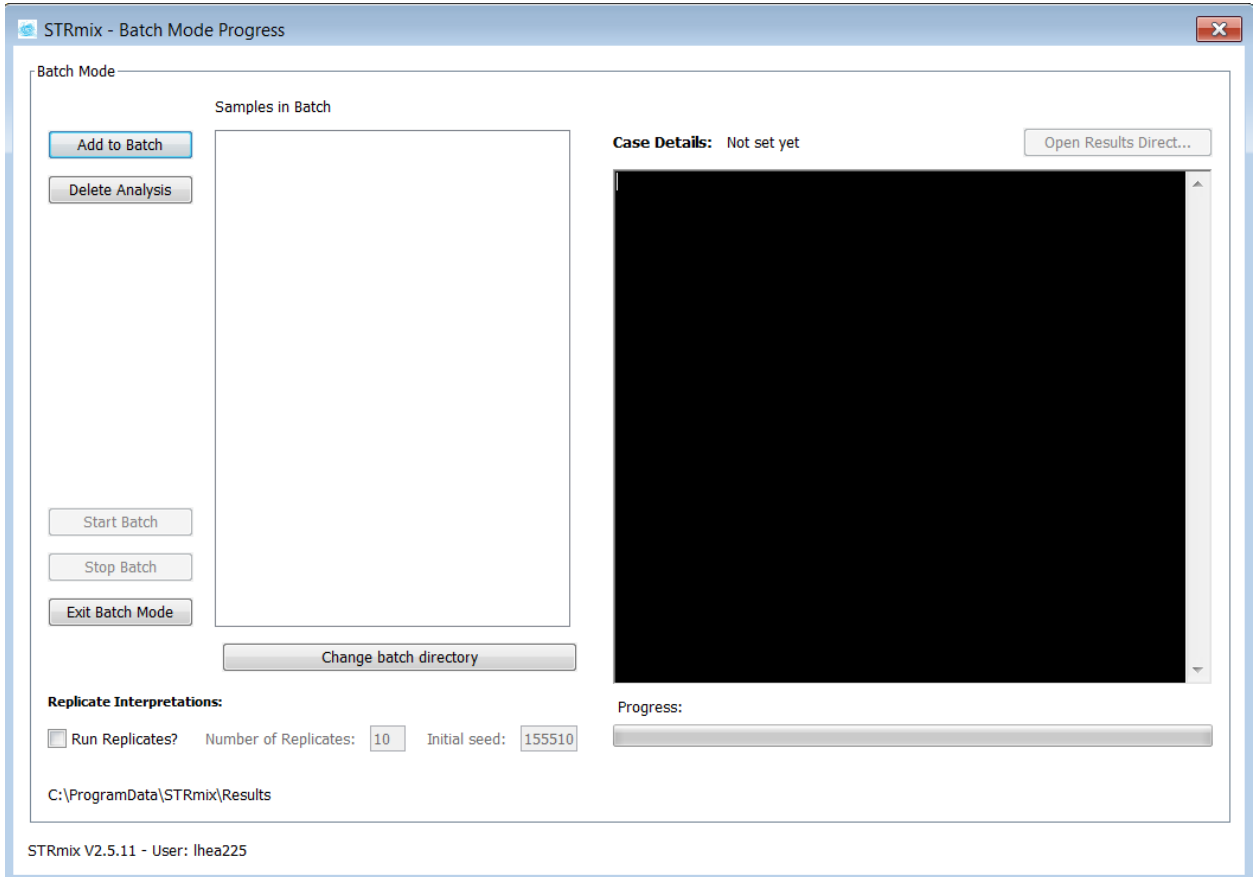


Figure 9

- Follow the same steps 2 through 8 (as needed) outlined in the “Single Sample Analysis” method above. Clicking the “Start” button at the end of step 8 will return you to the Batch Mode Progress window.
- Repeat the Add to Batch process until all desired samples are shown in the “Samples in Batch” area. Click the “Start Batch” button. Individual results files for each sample will be found in the STRmix™ Results folder.

STRMIX™ DATABASE SEARCHING

STRmix™ has a database searching feature which offers the option of searching an unresolvable mixture against a database in Excel format. Each of the individuals in the database are considered as a potential contributor in turn to the mixture under the following two hypotheses:

H_p: Database individual and N - 1 unknown contributors

H_d: N unknown contributors

where N is the number of contributors under consideration, as set by the analyst in the STRmix™ mixture analysis. The likelihood ratios in this feature are run without accounting for theta (FST) to improve run time. Individuals giving an LR above some list management value are investigated. A suggestion is to use 1000 to reduce the risk of adventitious hits.

The WSP elimination DNA database has been formatted for use with the STRmix Database Search feature. All unidentified deconvolution components suitable for comparisons will be searched against the

WSP elimination DNA database. If eligible, the profile may also be searched against CODIS.

There are two options for searching this database within STRmix™.

Option 1 (previously deconvoluted mixture)

1. From the STRmix™ main menu, select “Database Search”.
2. Drag the entire results folder from the original deconvolution into the Analysis File box (see Figure 10).

Figure 10

3. Select “Select Database”. Navigate to the WSP elimination database and select Open. Set the Minimum LR to 1000 and select “FBI_extended_Cauc” from the Population for Search drop down menu. Select “Standard” for Type of Search and set FST point estimate to 0.0. Click the “Save as kit defaults” button to save the settings for the next search session. Click “Start” to perform the search.
4. On completion of the search, select “Finish” to close the window. Results are automatically saved in a results folder once “Finish” is selected. Review the Database Search Report for any matches above the LR cutoff. If no matches are found, the report need not be printed, but the database search file shall be saved as part of the electronic case record data. If a match is returned and is determined to be related to a quality variance, the report shall be printed and included in the case file.

Option 2 (performed in conjunction with the deconvolution)

1. When setting-up either a single sample analysis or batch sample analyses, select “Start & Search” from the Populations Settings window (Figure 8).
2. Follow steps 3 and 4 from Option 1.

EVALUATION OF THE STRMIX™ RESULTS

STRmix™ performs deconvolutions of samples based on biological phenomena that have been well characterized. STRmix™ models DNA template amount and degradation for each contributor, locus amplification efficiencies across the DNA profile, as well as n-1 and n+1 repeat stutter. STRmix™ also takes into account sampling variation (i.e. heterozygote peak height balance) when assessing whether a possible answer could be true. Successful STRmix deconvolutions should be intuitive, in that the genotype pairings and weights should logically correspond with the DNA data.

An analyst review of the genotype pairings and weights remains the primary diagnostic for review of a STRmix™ mixture deconvolution.

There may be instances when the results obtained do not intuitively seem correct. Examples of this are:

- The mixture proportions do not reflect what is observed.
- The degradation does not reflect what is observed.
- The interpreted contributor genotypes do not appear intuitively correct.
- Large LR's (greater than 1) are obtained for each locus, except a small number where the LR is 0, and the relevant reference sample appears consistent with the evidence profile.

Causes for these issues include:

- The MCMC has not run for enough iterations.
- The number of contributors has not been correctly assigned.
- The data is of excessively poor quality (e.g. PCR inhibition or significant degradation).
- An artifact peak was included in the STRmix™ input file.

Potential solutions for these issues include:

- Perform additional work on the sample to try to improve data quality (e.g. re-amplify or re-extract).
- Re-run the STRmix™ deconvolution at an increased number of iterations (5,000,000 post burn-in accepts).
- Correct the STRmix™ input file.

If STRmix™ is run with increased number of iterations, the deconvolution and likelihood ratios from the higher iteration run will be reported, printed, and retained in the case file. Results from non-reported deconvolutions need only be retained in the electronic case record data.

If a STRmix analysis is inadvertently run using incorrect parameters, the results of the analysis do not need to be retained in the case record.

Additional diagnostics information within the STRmix™ deconvolution report may be used to determine if the deconvolution has successfully converged on the best MCMC sampling space:

1. Log (likelihood) – should be above zero. The larger this value, the better STRmix™ has been able to describe the observed data. A low or negative value suggests STRmix has not been able to describe the observed data given the information provided. In these instances further analyst scrutiny of the profile and deconvolution is appropriate. Reasons for a low or negative value include:
 - a. The profile is simply very low level and there is very little data making up the likelihood value.

- b. The assigned number of contributors is wrong resulting in forced stochastic events in the STRmix™ run as a result (e.g. large heterozygote peak imbalances or variation in mixture proportions across the profile)
 - c. Data has been removed that was real, particularly stutter peaks, and must now be described in STRmix™ by dropout
 - d. Artifact peaks have been included in the STRmix™ input file and must now be accounted for in STRmix™ by drop-in.
2. Gelman-Rubin convergence diagnostic – should be less than 1.2. This value indicates how well the MCMC analysis chains converged on the final profile deconvolution. A higher value indicates the chains may not have converged during the run. An elevated Gelman-Rubin convergence diagnostic in conjunction with other unsatisfactory diagnostic indicators may be used to justify re-running the deconvolution using a higher number of iterations.
3. Allele and stutter variance constants – these values indicate how well the data has been explained using the STRmix biological models. If a variance constant (C^2 for allele variance and K^2 for stutter variance) has increased markedly from its respective mode, this may indicate that the data is not being explained well by the STRmix biological models. This issue may result when a DNA profile's quality is sub-optimal or when the number of assigned contributors is incorrect. Observed in conjunction with the low/negative log(likelihood) diagnostic, a large allele or stutter variance constant indicates the deconvolution requires further scrutiny. If the sample is simply low level a low log(likelihood) and variance constants close to the mode will likely result. If non-artifacts have been omitted, artifacts have been left in, or the profile was otherwise misinterpreted a low log(likelihood) and high allele and/or stutter variances relative to the mode could result.

Note: High Elevated Gelman-Rubin convergence diagnostic values and high allele variance constant values markedly increased from their mode can be caused by a known issue with the stutter associated modeling of with allele 14 at vWA. The true observed stutter may be significantly lower than what is modeled by STRmix. If investigation determines this is the cause of unintuitive genotype pairings or weightings and/or diagnostic values outside the expected range, the deconvolution shall be repeated with the vWA locus omitted.

If the STRmix deconvolution results in a fully deduced contributor (a single, unambiguous genotype at all loci in the “Component ≥99%” column), this will be considered a “discrete contributor” and may be identified as an individual (e.g. Individual A). Only deduced discrete contributors should be compared to other profiles in the case, manually or via calculation of a likelihood ratio as appropriate. If the same discrete contributor is observed in multiple samples, this “match” can be confirmed manually. All comparisons to discrete contributors shall be reported; however, a statistic will not be reported and the “LR From Previous” report will not be printed, but shall be retained in the electronic data.

An apparent, trace level contributor (e.g. only a few elevated stutter position peaks and a barely detectable Y-STR peak) with a single major contributor could cause STRmix to produce contributor proportion estimates that do not appear to be intuitively correct. In this case, consider deconvolution without counting a trace contributor in the NOC estimate. If the genotype appears to be intuitively correct, report a single major with a possible trace, uninterpretable contributor.

CALCULATION OF A LIKELIHOOD RATIO IN STRMIX™

LR FROM PREVIOUS METHOD

1. Select “LR From Previous” from the main menu.
2. Drag the deconvolution results folder to the “Previous Analysis” box and click “OK.”
3. Edit Sample ID or Case Notes section as desired. Low memory mode and loci to be ignored can be selected as previously described under the Single Sample Analysis instructions. Click the “Confirm” button to move to the Add Profile Data window.
4. The reference profile to be compared can be selected either by dragging the .txt file exported from GMID-X into the “Add Reference Profile Data” box and selecting the correct sample from the pop-up window, or by selecting the “Add Reference” button, followed by the “Add Profile” button in the resulting window, and selecting the correct file and sample under the “Import from plate text file” header. Click the “Add Profile Data” button, followed by the “Save Reference” button to return to the Add Profile Data window.
5. Multiple reference samples may be selected to accommodate various LR propositions, however generally each reference will be compared individually. Click the “Confirm Settings” button.
 - a. If more than one reference sample yields an inclusionary LR to the same evidence profile, it is recommended that a “combined LR” be calculated to determine if all such references can be included in the evidence profile together (e.g. Reference 1 + Reference 2; 2 unknowns).
 - b. If only two reference samples are included and this “combined LR” does not support their combined inclusion a comment shall be added to the report to reflect that, while both individuals are included, they cannot both be in the mixture.
 - c. If more than two reference samples are included and this “combined LR” does not support their combined inclusion consult with a supervisor, technical lead, or the DNA Technical Leader to determine the appropriate additional LR propositions to compare.
 - d. The “combined LR” report will be maintained only in the electronic data (not printed) and the statistic will not be reported.
6. Select the 3 populations to be used from the Population Setting drop down menu: FBI_extended_Cauc, FBI_extended_AfAm and FBI_extended_SW_Hisp. The Caucasian database should be selected first. Then click the “Add Population” button to add the remaining two databases (see Figure 11). Click the “Save as Default Populations” button so these options do not need be chosen for future analysis.

STRmix - Population Settings

Case Details: STRmix test duplicate - 3nsf STRmix-LRPrev Open Results Directory

Population Settings

FBI_extended_SW_Hisp Add Population Remove Population

Population	Proportion	FST	Allele Freq File
FBI_extended_Cauc	0.3333333333333333	0.01b(1.0,1.0)	FBI_extended_Cauc.csv
FBI_extended_AfAm	0.3333333333333333	0.01b(1.0,1.0)	FBI_extended_AfAm.csv
FBI_extended_SW_Hisp	0.3333333333333333	0.01b(1.0,1.0)	FBI_extended_SW_His...

Factor N!

☒ Factor of N! LR

Sampling Variation

☒ Calculate HPD ☒ Include MCMC uncertainty

HPD iterations: 1000 Quantile: 99 Sides: 1

Save as Default Populations Cancel Back Start Start & Search

STRmix V2.5.11 - User: ajag225

Figure 11

- Click the “Start” button to perform the likelihood ratio calculation.
- Results are automatically saved in the results folder once Finish is selected.
- The lowest 99% 1-Sided Lower HPD Interval likelihood ratio will be used to determine if the person of interest is included or, excluded, (see Reporting section).

LR BATCHER METHOD

The LR Batcher tool allows the user to calculate multiple LRs from multiple reference inputs to a previously run deconvolution and vice versa. Please note that the kits need to be the same for each deconvolution and the reference sample(s). Directions for how to use this can be found in the STRmix Operations Manual (page 27 for the 30/June/2017 edition).

REPORTING

The LR shall be assessed to determine if the person of interest is included, excluded, or can neither included nor excluded. If the LR is 1 the result is “uninformative”.

These values are based on the 2018 SWGDAM recommendations on Genotyping Results Reported as Likelihood Ratios and LR estimates for profiles at the limits of interpretation that were observed in validation.

- If the LR supports an inclusion, the numerical LR will be reported in words, truncated to 2 significant figures (e.g. 5.9 million (10^6)).

2. If the LR supports an exclusion:
 - a. If the LR is between 1 and 0.01 the statistic will be reported. This will be formulated in the conclusion as 1/LR and stated as in support of Hd.
 - b. If the LR is <0.01 just report an exclusion.
3. If the 99% 1-Sided Lower HPD Interval LR = 0, there are two possible explanations. Both should be evaluated before reporting the result.
 - a. If the majority of loci yield an LR >1.0, but a few result in an LR of zero. Those loci should be evaluated for input errors, incorrect number of contributors assumed, or if an intuitively valid genotype combination was not considered by STRmix™. Rectifying errors, changing the number of contributors, or increasing the number of iterations (Configure Analysis window, see Figure 1) may resolve these loci. Justification for these actions shall be documented in the case file.
 - i. If resolution is not achieved by the above options consult a Supervisor, Technical Lead, or the DNA Technical Leader.
 - b. If most loci yield a LR <1.0 and the final LR is zero, this will be reported as an exclusion.

DEDUCING PROFILES FOR CODIS ENTRY

STRmix™ can be used to assist in the deduction of a profile for CODIS entry.

1. The Component Interpretation section of the Deconvolution Report can be used.
2. For each contributor, if a single genotype is listed in the “Component ≥99%” column, that genotype may be entered into CODIS. The analyst should ensure that this genotype makes intuitive sense before entry into CODIS. If the analyst determines a different entry would be more appropriate to minimize the risk of missing a candidate match, the reason should be documented in the case file.
3. Where “X,F” (X being an assigned allele and F denoting an allele any) is listed in the “Component ≥99%” column, the assigned allele may be entered into CODIS in the Forensic Partial or Mixture category, with the Partial locus indicator set to “Yes.”
 - a. If this results in the profile not being eligible for NDIS, additional evaluation of the Component Interpretation information to determine if any additional alleles can be added shall be performed (see step b below).
 - b. The assigned allele can be obligated (X+) with up to 3 additional alleles based on the alleles assigned in the highest weighted genotypes that add up to ≥99.00%.
 - i. If this results in the inclusion of a “Q” or more than a total of 4 alleles, only the obligated allele shall be entered into CODIS.
4. Where “F,F” is listed in the “Component ≥99%” column, the alleles assigned in highest weighted genotypes should be considered for CODIS entry until a value of ≥99.00% is achieved.
 - a. If this results in the inclusion of a “Q” or more than a total of 4 alleles, this locus shall not be entered into CODIS.

Note: it may be possible to combine genotype combinations from two probative contributors to enter the combined profile as a Forensic Mixture. This is more likely to occur when the contributor proportions approach 1:1.

1. For CODIS searching purposes only, it may be appropriate to repeat a deconvolution of a profile and condition on a probative known individual or an unknown individual identified elsewhere in the case to deduce the a profile of another unknown individual for CODIS entry. Known individual - situations where the original deconvolution and subsequent comparison of the suspect reference sample has determined that the suspect is a possible contributor to a mixed semen profile from a sexual assault sample (LR above the uninformative range). It would then be possible to repeat the deconvolution of the profile, conditioning on the suspect, in an attempt to deduce another contributor for CODIS entry.
2. Unknown individual – situations where an unknown individual (e.g. Individual A) is identified on other items in the case and appears to also be a contributor to a mixed profile. The deconvolution of the mixed profile could be repeated, conditioning on Individual A, to attempt to deduce a profile for another unknown individual (e.g. Individual B) for CODIS entry. Individual A must have previously been identified in a single source sample or as a single genotype with a weight of $\geq 99.00\%$ at all loci in the component interpretation of another mixture.

If a profile is entered into an SDIS-only category and a potential hit to a convicted offender is obtained, the following process shall be followed prior to requesting match confirmation from the CODIS lab.

1. Create a reference input file using the convicted offender genotype on the Match Detail Report (see “Manual creation of files for STRmix™ input”).
2. Use the “LR from Previous” function to compare the convicted offender profile to the original mixture deconvolution.
3. The results shall be technically reviewed (to ensure input data and LR are correct) and the date and initials of the technical reviewer shall be documented on the LR from Previous Report.
 - a. If the lowest LR generated, is an inclusion or “supports exclusion” proceed with requesting match confirmation as appropriate.
 - b. If the lowest LR generated is an “exclusion”, the candidate match will be dispositioned as a “No Match” and will not be sent for match confirmation or reported.
 - i. If the convicted offender profile is not typed in Fusion 6C, the potential hit should be manually assessed for consistency in genotype weights regardless of the STRmix™ result. Consideration should be given to requesting the offender sample be re-typed in Fusion 6C
4. Documentation retained in the casefile shall be:
 - a. If the LR result is above the uninformative range, the Match Detail Report and LR from Previous Report shall be retained.
 - b. If the LR result is below the uninformative range, neither the Match Detail Report or the LR from Previous Report shall be retained.
 - c. If the LR result is in the uninformative range, a note shall be made in the comment section during dispositioning that the “No Match” was determined based on an uninformative LR from STRmix. The Match Detail Report shall not be included in the case file if the match is dispositioned as a no match.

CODIS & CODIS MATCH PREDICTION

Once peer reviewed, eligible STR profiles that meet the guidelines developed for CODIS use will be uploaded and searched. All forensic partial and forensic mixture profiles for CODIS use will be assessed for compliance with Moderate Match Estimation threshold requirements using the CODIS software. See the Washington State Patrol Crime Laboratory Division Convicted Offender/CODIS Program Standard Operating Procedures for guidelines. If an eligible profile is deemed unsuitable for SDIS and/or NDIS, the reason(s) will be documented in the case file.

Analysts may choose to de-convolve a mixture and search the components separately, even though the mixture may not meet the requirements for identifying major/minor components or for assumptions regarding an endogenous DNA profile. An analyst may use their best judgment to pick out a stronger or lesser contributor or use additional assumptions to remove alleles from a mixture. Obligate allele designation can also be used. When searching mixture components, analysts will ensure that straddle or dropout will not cause a 'no match' condition. For de-convolved mixtures, the Match Estimation Tool in the CODIS software may be utilized to help assess the discriminating power of each component to reduce the number of adventitious matches. See the Washington State Patrol Crime Laboratory Division Convicted Offender/CODIS Program Standard Operating Procedures for guidelines. The decision to search a de-convolved component of a mixture will be made on a case-by-case basis and will involve agreement between the analyst and the technical reviewer. Any CODIS match to a complex mixed profile shall be evaluated based on the peak height ratios, contributor ratios, and assumptions initially used to evaluate the profile. If it is not possible to make exclusions to the profile, then the profile is considered not interpretable and is not CODIS eligible.

It may be beneficial to upload/search sub-threshold alleles at the SDIS level and, if eligible, the NDIS level of CODIS. A decision to search sub-threshold alleles will be made on a case-by-case basis and will involve agreement between the analyst and peer reviewer.

SAMPLE SWITCH DETECTION PROCEDURE

The primary method used to avoid sample switches is to ensure that all samples within a batch shall have easily discernable unique identifiers. This applies to both reference and questioned samples.

At least one, or a combination of more than one, of the following sample switch detection protocols must be used if there is a need to process two or more reference samples from the same case and of the same gender:

1. STR confirmatory sample (SCS: DNA from a case reference sample previously run) - If two or more probative reference samples of the same gender are processed together for a case and one of them results in a match to the evidence, the analyst will go back to the reference sample that matched and take a second cutting. This second cutting will be typed by the case analyst or another DNA analyst to verify the match. The original match and the reference verification will both be part of the peer review, and will be done **before the report can be released**. Alternatively, a second cutting may be taken during the initial processing of one of the samples and run in the same batch if only two probative reference samples of the same gender are processed together for a case. A match does not need to occur for an analyst to use the SCS program.
2. Witnessing – The witnessing step entails checking both the sample name and case number. If two or more probative reference samples of the same gender are processed together for a case, all reference processing steps of the case analyst will be witnessed by a DNA staff member, except for quantification. This includes the preparation of reference sample (e.g. cutting), sample labeling and order of processing (manual tube or well setups and transfers) from DNA extraction to final loading for CE injection. Each step of the process that is witnessed will be indicated by the initials of the witnessing DNA staff member in the case file notes. The identifiers must be checked carefully during witnessing. If a multi-channel pipette is used to transfer from an amp plate or strip tubes, no witnessing is necessary.
3. Separate Analysis - If two or more probative reference samples of the same gender exist for a case, the analyst, or different analysts, will conduct separate analyses for each reference sample. Separate analyses includes different start times for the sampling of the reference sample material, separate extraction times (robotic or manual) in separate batches to include separate reagent blanks, and separate amplification/CE load times.

NOTE: If a combination of more than one approach is employed, a confirmation sample or sampling (similar to SCS) need only occur from the last witnessed or separately analyzed processing step.

GUIDELINES FOR REPORT WRITING

The procedures for casework reports are outlined in the WSP CLD Operations Manual and in the WSP DNA Analysis Quality Assurance Manual, Reports and the Release of Information section.

1. Items Examined – There shall be a list and description of evidentiary items examined. Also items that were received but not examined should be mentioned. An example statement would be, “No examination of the remaining items (i.e. listed by number) was conducted.”
2. Methods and Observations – A description of the types of forensic technology used and/or analyses conducted.
3. Results and Conclusions – Results and conclusions are presented. These can be optionally expressed in a tabular format.
4. Remarks – Place for item disposition detail and any other appropriate statements of information to be disclosed in the report.

GENERIC STATEMENTS

A description of the DNA analysis shall include the genetic markers used or the amplification kit used, or both.

- Example: PowerPlex® Fusion 6C is a Short Tandem Repeat (STR) multiplex which tests the following genetic regions: D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, SE33, D22S1045, DYS391, FGA, DYS576, DYS570, and Amelogenin (a sex determination site).

Statistical calculations were computed by CODIS Popstats. Or Statistical calculations were computed by STRmix™ interpretational software (if statistics are being reported). [Full citations can be added; refer to the Reference section of this manual].

STRmix™ interpretational software was used to aid in the analysis of the DNA profiles obtained (if STRmix™ is used, but no statistics are reported).

When there is more than one item/sample with a matching DNA profile to a known sample and they differ in the number of loci identified, the following statement can be added to explain the different random match probability estimates given. For example: The significance of the association increases with the number of genetic loci (or markers) involved in determining the match.

For reports involving LR inclusions, verbal conventions may be used to express the support or strength of the likelihood ratio.

SWGDM (2018)* recommends the likelihood ratio ranges and terms provided in the following table.

Scale of verbal qualifiers for reporting likelihood ratios

LR for Hp Support and 1/LR for Hd Support	Verbal Qualifier
1	Uninformative
2 – 99	Limited Support
100 – 9,999	Moderate Support
10,000 – 999,999	Strong Support
≥1,000,000	Very Strong Support

* RECOMMENDATIONS OF THE SWGDAM AD HOC WORKING GROUP ON GENOTYPING RESULTS REPORTED AS LIKELIHOOD RATIOS (2018)

GUIDELINES FOR CONCLUSION STATEMENTS – SEMI-QUANTITATIVE BINARY APPROACH

It may be appropriate to add "...including at least X male contributors" to the conclusion statements.

SINGLE CONTRIBUTOR PROFILE

Inclusion

The DNA typing profile obtained from (biological material and item description) item..., matches that of the known sample, item ...(donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x.

Exclusion

The donor of the known sample, item ...(donor's name) is not the source of the DNA obtained from (description), item...(option #1)

The donor of the known sample (donor's name) is excluded as a possible source of the DNA obtained from (description), item...(option #2)

Inconclusive

The DNA typing profile (or the trace DNA typing profile) obtained from (description) item..., is of limited genetic information and no meaningful comparison can be made. (option #1)

No (or no interpretable) DNA typing profile was obtained from (description) item.... (option #2)

No forensically significant DNA typing profile was obtained from (description) item.... (option #3 – Include case specific detail to explain the uninformative result.)

Unknown Individual

The DNA profile obtained from (description) item..., is from an unknown (male/female) source designated as Individual A. (option #1)

The DNA profiles from (description) item... and (description) item... match and this profile is designated as being from an unidentified (male/female), Individual (or source) A. (option #2)

The DNA profiles from (description) item... and etc. originate from an unidentified (male/female) source designated as Individual A. (option #3)

MIXTURES – ASSUMING PRESENCE OF AN EXPECTED DNA PROFILE

Inclusion

RMP (Type 1, Type 2, and Type 4 Mixture)

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from two (or more) individuals. Assuming that the donor of the known sample, item...(donor) is one of the sources of the DNA from this profile, then the other component matches the profile obtained from the donor of the known sample, item...(donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x.

LR

Hypothesis: Two known contributors vs. one known and one unknown (e.g.: intimate sample from victim)

A mixed DNA profile was obtained from the (description) item. The DNA profile present is consistent with the combined known profiles from [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [victim] and [suspect] than it having originated from the [victim] and an unrelated individual selected at random from the U.S. population.

A mixed DNA profile was obtained from the (description) item. The DNA profile present is consistent with originating from the known profiles of [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [victim] and [suspect] than it having originated from the [victim] and an unrelated individual selected at random from the U.S. population.

(Option #2)

Add “major contributors mixture” as needed for mixtures with distinct major mixture.

Exclusion

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from two individuals. Assuming that the donor of the known sample, item...(donor) is one of the sources of the DNA from this profile, then the other component is that of an unknown male (female) individual. The donor of the known sample, item...(donor) is not the (or can be excluded as the) source of this unknown profile.

MIXTURES – DIFFERENTIAL EXTRACTION

Inclusion

RMP (Type 1 mixture)

The DNA typing profile obtained from the semen on (or male component of) (description) item ...matches that of the known sample, item .. (donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x. The profile of the female component matches that of the known sample, item .. (donor). (option #1)

A DNA profile matching that of the known sample, item.. (donor) was obtained from ... (variation of option #1)

The DNA typing profile obtained from (description) item .. is of mixed origin consistent with having originated from two individuals. The profile of the semen/male component matches that of the known sample, item ..(donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x. The profile of the female component matches that of the known sample, item .. (donor). (option #2).

LR

Hypothesis: Two known contributors vs. one known and one unknown (e.g.: #1 intimate sample from victim where subtraction of the victim leads to ambiguity in the deduced profile or #2 non-intimate sample when the presence of both victim and suspect is probative and there is profile ambiguity between the differential components)

A DNA typing profile was obtained from the semen on (or male component of) (description) item. The DNA profile present is consistent with the combined known profiles from [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the

[victim] and [suspect] than it having originated from [victim] and an unrelated individual selected at random from the U.S. population.

Add “non-sperm or female component” as needed.

Add “major contributors mixture” as needed for mixtures with distinct major mixture.

Add “significant contributors” as needed for mixtures with a distinct mixture from two significant contributors with one or more non-interfering trace contributors.

Exclusion

The donor of the known sample, item ...(donor) is not the source of the semen/male DNA obtained from (description) item.... (option #1)

The donor of the known sample, item ...(donor) is excluded as the source of the semen/male DNA obtained from (description) item....(option #2)

Inconclusive

Statements of inconclusive results described previously can be adapted where appropriate.

MIXTURES –WITH MAJOR COMPONENT

Inclusion – Major Component

RMP (Type 1 Mixture)

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from at least two individuals. The profile of the major component matches that of the known sample, item...(donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x.

RMP (Type 3 Mixture)

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from at least N individuals. The profile of the major component matches that of the known sample, item...(donor). The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x.

LR (Type 4 Mixture)

Hypothesis: One known contributor and one unknown vs. two unknowns (e.g.: bloodstain at scene)

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from at least N individuals. A major component consistent with two individuals was detected. [Donor (item #)] is included as one of the possible sources of the major component obtained from this item. It is # times more likely that the observed major component occurred as a result of a mixture of the [donor] and an unknown individual than if it originated from two unrelated individuals selected at random from in the U.S. population.

Hypothesis: Two known contributors vs. one known and one unknown (e.g.: bloodstain on suspect’s shirt)

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from at least N individuals. A major component consistent with two individuals was detected. This major component is consistent with the combined known profiles from [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed major component occurred as a result of a mixture of

the [victim] and [suspect] than it having originated from [victim] and an unrelated individual selected at random from the U.S. population.

Inclusion – Minor Component

LR (Type 1 Mixture)

Hypothesis: One known contributor and one unknown vs. two unknowns (e.g.: bloodstain at scene)

A mixed DNA profile consistent with originating from 2 individuals was obtained from the (description) item. [Donor (item #)] is included as one of the possible sources of the DNA profile obtained from this item. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [donor] and an unknown individual than if it originated from two unrelated individuals selected at random from in the U.S. population.

Hypothesis: Two known contributors vs. one known and one unknown (e.g.: bloodstain on suspect's shirt)

A mixed DNA profile was obtained from the (description) item. The DNA profile present is consistent with the combined known profiles from [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [victim] and [suspect] than it having originated from [victim] and an unrelated individual selected at random from the U.S. population.

Exclusion

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from two (or more) individuals. The donor of the known sample, item...(donor) is excluded as (or is not) a contributor to this profile.

Inconclusive - Trace Component

No meaningful comparisons can be made to the trace DNA of limited genetic information obtained from (description) item.... (option #1)

MIXTURES –WITHOUT A MAJOR COMPONENT

Inclusion

LR

Hypothesis: One known contributor and one unknown vs. two unknowns (e.g.: bloodstain at scene)

A mixed DNA profile consistent with originating from two individuals was obtained from the (description) item. [Donor (item #)] is included as one of the possible sources of the DNA profile obtained from this item. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [donor] and an unknown individual than if it originated from two unrelated individuals selected at random from in the U.S. population.

Hypothesis: Two known contributors vs. one known and one unknown (e.g.: bloodstain on suspect's shirt)

A mixed DNA profile was obtained from the (description) item. The DNA profile present is consistent with the combined known profiles from [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [victim] and [suspect] than it having originated from [victim] and an unrelated individual selected at random from the U.S. population. (option #1)

A mixed DNA profile was obtained from the (description) item. The DNA profile present is consistent with originating from the known profiles of [victim (item #)] and [suspect (item #)]. It is # times more likely that the observed DNA profile occurred as a result of a mixture of the [victim] and [suspect] than it having originated from [victim] and an unrelated individual selected at random from the U.S. population. (option #2)

Exclusion

The DNA typing profile obtained from (description) item... is of mixed origin consistent with having originated from two (or more) individuals. The donor of the known sample, item...(donor) is excluded as (or is not) a contributor to this profile.

Inconclusive

The partial DNA typing profile obtained from (description) item... is of mixed origin consistent with three (or more) individuals. Due to the complexity of the DNA profile, no meaningful comparison can be made to any known samples. (Option #1 - for multiple contributors, i.e. more than 3)

Due to the complexity of the mixture and the (possible) presence of a trace contributor, no conclusion can be made regarding the inclusion or exclusion of (Option #2)

SUMMARY TABLES

To be assessed on a case by case basis, it may be appropriate to present results from several samples by using a summary table such as the examples following.

Summary of Screening Results

Item #	Description	Screening Result
1	Jeans – suspect	blood identified
2	Stain/swab – scene	presumptive for blood

Summary of DNA Match Results

Item #	Description	Match	Probability ¹
1	Bloodstain - jeans	Item A (donor)	1 in 25 billion

¹The estimated probability of selecting an unrelated individual at random from the U.S. population with a matching profile is 1 in x.

REMARKS STATEMENTS

The following are examples of the types of statements that could be placed in this section of the report. Exhibit Disposal could also be a separate heading or be included in this section.

1. All remaining items of evidence will be returned to the submitting agency (will be retained at this laboratory pending return to the submitting agency).
2. A glossary of terms.

3. A DNA typing profile attributable to the analyst was identified on a sample from item A. This is not forensically significant to the conclusions made in the analysis of the evidential material.
4. A DNA typing profile attributable to the analyst was identified on a sample from item A. This was considered in the forming of conclusions made from the analysis of the evidential material.
5. A DNA typing profile attributable to an elimination sample (e.g. WSP Crime lab Division staff) was identified on a sample from item A. This is not forensically significant to the conclusions made in the analysis of the evidential material.
6. A DNA typing profile attributable to an elimination sample (e.g. WSP Crime lab Division staff) was identified on a sample from item A. This was considered in the forming of conclusions made from the analysis of the evidential material.
7. Only the most conservative estimate from the data base populations used is reported for the statistical assessment.
8. DNA work product from Items X and Y was packaged as a newly created item (new item number) for return to the submitting agency.

GUIDELINES FOR CONCLUSION STATEMENTS – STRMIX™ APPROACH

Profile exceeds computational power

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. An attempt was made to analyze this mixture with the STRmix™ interpretational software. The results exceed the computing capacity of the software and are unsuitable for comparisons.

Uninterpretable Mixtures (more than 8 alleles at one or more loci)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from at least 5 individuals. Due to the complexity of the mixture, this profile is not suitable for interpretation or comparisons.

Single Contributor Profile

Inclusion

The single source DNA typing profile obtained from [biological material and item description] (item #), matches [name] (item #). It is # times more likely to observe this DNA profile if [name] is the donor rather than an unrelated individual selected at random from the U.S. population.

LR in Uninformative Range (LR=1)

The single-source DNA profile obtained from [biological material and item description] (item #) is suitable for comparisons. However, [Name] can be neither included nor excluded as the donor of this profile. It is equally likely to observe this DNA profile if [Name] is the donor rather than an unrelated individual selected at random from the U.S. population.

LR supports exclusion (e.g. LR=0.01 to 1; numerically reported as a value between >1 and 100)

The DNA obtained from [biological material and item description] (item #) is suitable for comparisons. It is “x” times more likely to observe this DNA profile if an unrelated individual selected at random from the U.S. population is the donor rather than [Name].

LR is an exclusion (LR<0.01)

The DNA obtained from [biological material and item description] (item #) is suitable for comparisons. [Name] is excluded as a contributor to this profile.

Unknown Individual

The DNA profile obtained from [description] (item #), is from an unknown (male/female) source designated as Individual A. (option #1)

The DNA profiles from [description] (item #) and [description] (item #) match and this profile is designated as being from an unidentified (male/female), Individual (or source) A. (option #2)

The DNA profiles from [description] (item #) and etc. originate from an unidentified (male/female) source designated as Individual A. (option #3)

Mixtures – Assuming Presence of Expected DNA Profile

Inclusion

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. [Assumed individual] and [Probative individual] are included as possible contributors. Assuming [NOC] contributors and [assumed contributor] as one of those contributors, it is # times more likely that the observed DNA profile occurred as a result of a mixture of the [assumed individual] and [probative contributor] [and x number of unknown contributors, as appropriate] than if it originated from [assumed individual] and [NOC-1] unrelated individual(s) selected at random from the U.S. population.

For samples that were extracted differentially, conclusions are required for each fraction. Add “non-sperm,” “semen,” “male component,” or “female component” as needed. A non-probative inclusion or fraction shall be reported without statistical analysis (e.g. “The female component of the vaginal swabs (item #) matches [donor of vaginal swabs].”).

LR in Uninformative Range (LR=1)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. Assuming [assumed contributor] is one contributor, the remaining component is from an/[number] unknown [male/female] individual(s) and is suitable for comparison. [Probative contributor] can be neither included nor excluded as the donor of this profile. It is equally likely to observe this DNA profile if [Probative contributor] is a donor rather than an unrelated individual selected at random from the U.S. population.

LR supports exclusion (e.g. LR=0.01 to 1; numerically reported as a value between >1 and 100)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. Assuming [assumed contributor] is one contributor, the remaining component is from an/[number] unknown [male/female] individual(s) and is suitable for comparison. It is “x” times more likely to observe this DNA profile if an unrelated individual selected at random from the U.S. population is a donor rather than [Name].

LR is an exclusion (LR<0.01)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. Assuming [assumed contributor] is one contributor, the remaining component is from an/[number] unknown [male/female] individual(s) and is suitable for comparison. [Name] is excluded as a contributor to this profile.

Mixtures –No Assumable Component

Inclusion

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals. *[Probative individual] is included as a possible contributor. Assuming [NOC] contributors, it is # times more likely that the observed DNA profile occurred as a result of a mixture of the [probative individual] and [NOC-1 number of unknown contributors] than if it originated from [NOC] unrelated individual(s) selected at random from the U.S. population.

Repeat from * onwards as needed for additional probative possible contributors.

LR in Uninformative Range (LR=1)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals and is suitable for comparisons. [Probative contributor] can be neither included nor excluded as the donor of this profile. It is equally likely to observe this DNA profile if [Probative contributor] is a donor rather than an unrelated individual selected at random from the U.S. population.

LR supports exclusion (e.g. LR=0.01 to 1; numerically reported as a value between >1 and 100)

The DNA typing profile obtained from [biological material and item description] (item #) is of mixed origin consistent with having originated from [NOC] individuals and is suitable for comparisons. It is “x” times more likely to observe this DNA profile if an unrelated individual selected at random from the U.S. population is a donor rather than [Name].

LR is an exclusion (LR<0.01)

The DNA obtained from [biological material and item description] (item #) is suitable for comparisons. [Name] is excluded as a contributor to this profile.

Mixtures with included contributors that cannot all be included in the mixture together

Add: However, [POI1] and [POI2] cannot both be included in the mixture together.

GUIDELINES FOR PATERNITY/KINSHIP

Interpretation of STR Profiles – Paternity, Parentage, and Kinship Determinations

In cases involving a disputed paternity, there may be a requirement to provide a paternity calculation where the mother's, child's, and alleged father's genotypes are known. In cases involving a disputed identity, there may be a requirement to provide a parentage calculation where the mother's, alleged child's, and father's genotypes are known. In both applications, the data is initially assessed for an inheritance pattern. A paternity example is provided:

First the data from the mother and child should be examined to determine the alleles contributed by the true father. For all loci, the mother and child should share one or both alleles.

In the case where the mother and child share one allele, then the allele found in the child but not the mother is from the true father.

In the case where the mother and child are homozygous, then this is also the allele received from the true father.

In the case where both mother and child are heterozygous and share both alleles, then it cannot be determined which was received from the true father and either could have been.

In a paternity case, the allele data from the alleged father should be examined to see if he carries the child's paternal alleles. If he does not, then he can be eliminated as the father. There is one caveat due to the mutation rates of STR loci: exclusion at more than 2 loci is required before an elimination conclusion can be declared due to the possibility of mismatches caused by mutation.

If an elimination conclusion cannot be declared, then a statistical evaluation of paternity or parentage can be reported. The effect of mismatches due to possible mutation will be included in the calculations. Following the American Association of Blood Banks (AABB) method, this can be accomplished by calculating the likelihood ratio at the effected locus/loci using the estimation of the mutation rate and mean power of exclusion.

For the PowerPlex Fusion 6C amplification system kit results, use either D12S391 or vWA in the PopStats calculation as it does not have the capability to account for possible linkage. Use whichever locus has the stronger statistical weight.

Calculating Probability of Paternity and/or Parentage using FBI POPSTATS

1. Select Parentage from the Popstats Calculations menu.
2. In the Configuration menu select Statistics Options... and choose Miscellaneous. Ensure that the prior probability is = 0.5.
3. Select trio for an alleged parent scenario or select Reverse for a missing child scenario. Enter all of the alleles for each locus that is to be used for the calculation.
4. Select Calculate.
5. Use the default mutation rate and mean power of exclusion.
6. Select Print from the file menu and print the parent/reverse parentage.

Calculating Probability of Kinship or Single Parentage using FBI POPSTATS

1. Select Kinship from the Popstats Calculations menu.
2. Enter all of the alleles for each locus that is to be used for the calculation.
3. Select all the appropriate kinship relationships to test.
4. Select Calculate Statistics from the Profile menu.

5. Enter the mutation rate and mean probability of exclusion for any locus mismatches for Parent Offspring calculation.
6. Select Print Report from the file menu and print Kinship Statistics Summary (or Locus for additional detail) or Single Parentage as appropriate.

Guidelines for Report Writing – Paternity, Parentage, and Kinship

GENERIC STATEMENTS

A description of the DNA analysis shall include the genetic markers used or the amplification kit used, or both.

- Example: PowerPlex® Fusion 6C is a Short Tandem Repeat (STR) multiplex which tests the following genetic regions: D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, SE33, D22S1045, DYS391, FGA, DYS576, DYS570, and Amelogenin (a sex determination site).

Statistical calculations were computed by CODIS Popstats.

Paternity/kinship inclusion reports shall include an appropriate weight of evidence statement

SWGDAM (2018)* recommends the likelihood ratio ranges and terms provided in the following table.

Scale of verbal qualifiers for reporting likelihood ratios

LR for Hp Support and 1/LR for Hd Support	Verbal Qualifier
1	Uninformative
2 – 99	Limited Support
100 – 9,999	Moderate Support
10,000 – 999,999	Strong Support
≥1,000,000	Very Strong Support

* RECOMMENDATIONS OF THE SWGDAM AD HOC WORKING GROUP ON GENOTYPING RESULTS REPORTED AS LIKELIHOOD RATIOS (2018)

Guidelines for Conclusion Statements – Paternity, Parentage, and Kinship

PATERNITY STATEMENTS

Inclusion of Suspected Father

It is x times more likely that the genetic testing results obtained would be observed if the donor of the known sample, item A (alleged father) is/was the biological father of the donor of item B (child/fetus/fetal tissue) rather than an unrelated individual selected at random from the U.S. population. A likelihood ratio greater than 1,000,000 indicates extremely strong evidence to support paternity.

In addition to the statistics reference and the weight of evidence conventions, include the following:

The probability of paternity estimate is (state %). The probability of paternity is a measure of the strength of one's belief in the hypothesis that the tested man is the father based on both the genetic evidence and non-genetic evidence. The laboratory did not consider any information about the other evidence in the case and assumed the non-genetic evidence is equivocal 50% one way or the other. Note: Refer to

Statistical Interpretation of STR DNA Typing section for probability of paternity estimate rounding requirements.

Inclusion and a Single Locus Mismatch

The following is used in addition to the above inclusion statement in the case of a single locus or two loci exclusion.

To determine a non-paternity relationship by STR analysis, exclusion at more than two loci is necessary. In this particular case, there was a mismatch at (state locus) that could be due to mutation. The mutation rate and mean power of exclusion from this locus were included in calculating the likelihood of paternity.

Exclusion (More Than Two Loci)

The donor of the known sample, item A (alleged father) is/was not the biological father of the donor of item B (child/fetus/fetal tissue).

PARENTAGE STATEMENTS

Inclusion of Unknown Child

It is x times more likely that the genetic testing results obtained would be observed if the donor of item A (alleged child) is/was the biological child of the donors of the known samples item B (mother) and item C (father) rather than an unrelated individual selected at random from the U.S. population. A likelihood ratio greater than 1,000,000 indicates extremely strong evidence to support parentage

Inclusion and a Single Locus Exclusion

The following is used in addition to the above inclusion statement in the case of a single locus exclusion.

To determine a non-parentage relationship by STR analysis, exclusion at more than two loci is necessary. In this particular case, there was a mismatch at only (state locus) that is presumed to be due to mutation. The mutation rate and mean power of exclusion from this locus was included in calculating the likelihood of parentage.

Exclusion (More Than Two Loci)

The donor of the known sample, item A (alleged child) is/was not the biological child of the donors of item B (donor) and item C (donor).

KINSHIP STATEMENTS

Inclusion of Missing Person Relative

Siblings

It is x times more likely that the genetic testing results obtained from item A (evidence) would be observed if the source of the profile is/was a biological sibling of the donor of the known sample item B rather than an unrelated individual selected at random from the U.S. population. A likelihood ratio greater than 1,000,000 indicates extremely strong evidence to support kinship.

Single Parent or Motherless Paternity

It is x times more likely that the genetic testing results obtained from item A (evidence) would be observed if the source of the profile is/was a biological child of the donor of the known sample item B rather than an unrelated individual selected at random from the U.S. population. A likelihood ratio greater than 1,000,000 indicates extremely strong evidence to support paternity/maternity.

Single Parent or Motherless Paternity Inclusion and a Single Locus Mismatch

The following is used in addition to the above inclusion statement in the case of a single locus or two loci exclusion.

To determine a non-paternity relationship by STR analysis, exclusion at more than two loci is necessary. In this particular case, there was a mismatch at (state locus) that could be due to mutation. The mutation rate and mean power of exclusion from this locus were included in calculating the likelihood of paternity.

Exclusion

Siblings

The source of the profile, item A (evidence) is/was not the biological sibling of the donor of item B (donor).

Single Parent or Motherless Paternity (More than 2 loci)

The source of the profile, item A (evidence) is/was not the biological child of the donor of item B (donor).

REMARKS STATEMENTS

The following are examples of the types of statements that could be placed in this section of the report. Exhibit Disposal could also be a separate heading or be included in this section.

- All remaining items of evidence will be returned to the submitting agency (will be retained at this laboratory pending return to the submitting agency).
- A glossary of terms.
- A DNA typing profile attributable to the analyst was identified on a sample from item A. This is not forensically significant to the conclusions made in the analysis of the evidential material.
- A DNA typing profile attributable to the analyst was identified on a sample from item A. This was considered in the forming of conclusions made from the analysis of the evidential material.
- A DNA typing profile attributable to an elimination sample (e.g. WSP Crime lab Division staff) was identified on a sample from item A. This is not forensically significant to the conclusions made in the analysis of the evidential material.
- A DNA typing profile attributable to an elimination sample (e.g. WSP Crime lab Division staff) was identified on a sample from item A. This was considered in the forming of conclusions made from the analysis of the evidential material.
- Only the most conservative estimate from the data base populations used is reported for the statistical assessment.
- DNA work product from Item X was packaged with the evidence.
- DNA work product from Items X and Y was packaged as a newly created item (new item number) for return to the submitting agency.
- Likelihood ratio values >100 in support of the defense proposition were reported as an exclusion.

REPORTS INVOLVING CODIS

If a DNA profile is searched as a one-time event, then a report must be generated clearly stating the results of the search. If a DNA profile is entered into a database(s) and is repeatedly searched with negative results, a report is not required for each search. However, the first time the profile is entered into the database, a report must be generated which clearly informs the customer that the profile was entered into the database. A report or follow-up report must also be generated anytime a positive association is made with the entered DNA profile (with the exception of conviction matches). Positive associations involving a solved case(s) in which the agency representative is the same can be reported by issuing one report only. Both case files will retain the necessary CODIS paperwork and the analyst not generating the report will indicate in their documentation which laboratory request contains this reported information.

If a match/hit to the CODIS database occurs, a statistical estimate is not provided until the match is confirmed by the analysis of a casework reference sample from the offender, submitted under proper chain of custody.

Note: In the absence of a WA State ID Number, another identifier may be used.

CODIS RELATED STATEMENTS – UNDER CONCLUSIONS SECTION

No Hit

The DNA typing profile obtained from (biological material and item description or unknown Individual A) item...was entered into the Combined DNA Index System (CODIS) and searched. No matches were found.

OFFENDER HIT Single Source

The DNA typing profile obtained from (biological material and item description or unknown Individual A) item..., was entered into the Combined DNA Index System (CODIS) and searched. A match between the DNA typing profiles of unknown individual A and (the offender's name) (date of birth, identifying number such as a State ID or FBI number) was declared. It is requested that a known reference sample from (the offender's name) be submitted to the Washington State Patrol (location) Crime Laboratory to confirm this match.

Forensic Mixture

The mixed DNA typing profile obtained from (biological material and item description) item.... was entered into the Combined DNA Index System (CODIS) and searched. (The offender's name) (date of birth, identifying number such as a State ID or FBI number) was determined to be a possible contributor to this profile. It is requested that a known reference sample from (the offender's name) be submitted to the Washington State Patrol (location) Crime Laboratory for confirmation purposes.

CASE TO CASE HIT Single Source to Single Source

The DNA typing profile obtained from (biological material and item description or unknown Individual A) item... was entered into the Combined DNA Index System (CODIS) and searched. A match between the DNA typing profile of unknown Individual A and a DNA typing profile generated from item(description) in (police agency, agency case number) was declared. Should any additional information be requested,

(agency representative's name) from (police agency) can be contacted at (phone number and/or email address).

Single Source to Forensic Mixture; Forensic Mixture to Forensic Mixture

The mixed DNA typing profile obtained from (biological material and item description) item... was entered into the Combined DNA Index System (CODIS) and searched. A forensic association between the DNA typing profile generated from item...(description) and a DNA typing profile generated from item(description) in (police agency, agency case number) was obtained. Should any additional information be requested, (agency representative's name) from (police agency) can be contacted at (phone number and/or email address).

CODIS RELATED STATEMENTS – UNDER REMARKS SECTION

Routine, scheduled searches of CODIS will be conducted. If a potentially probative match should occur, a subsequent report will be issued.

MATCH WITH A SAMPLE FROM THE ELIMINATION DATABASE

The DNA typing profile obtained from (biological material and item description or unknown Individual A) was searched against the state level of the Combined DNA Index System (CODIS) and a match to a profile in the elimination database was obtained. The elimination database contains DNA profiles from WSP Crime Lab Division staff, law enforcement representatives, and other known individuals. The DNA typing profile obtained from (biological material and item description or unknown Individual A) was removed from the CODIS.

ONETIME SEARCH

NO HIT

A onetime search of the DNA typing profile obtained from the reference sample of (suspect name) item... was performed against the state level of the Combined DNA Index System (CODIS) and no probative matches were found.

HIT

A onetime search of the DNA typing profile obtained from the reference sample of (suspect name) item ... was performed against the state level of the Combined DNA Index System (CODIS) and a match occurred to a DNA typing profile generated from item...(description) in (police agency, agency case number). Please refer to Washington State Patrol (location) Crime Laboratory report (case number) dated (date) issued by forensic scientist (name).

Should any additional information be requested, (agency representative's name) from (police agency) can be contacted at (phone number and/or email address).

CODIS EXPORT

To successfully execute this procedure, the analysis of data and creation of the CODIS export file must be done using a GMIDx login that is the same as the user's CODIS login name.

ESTABLISH SOURCE AND DESTINATION LABS FOR CODIS EXPORT FILES

- 1.) In GMIDx, go to Tools > CODIS Export Manager.
- 2.) Under Source Lab IDs, enter the source lab ORI and select 'add'.
- 3.) Under Destination Lab IDs, enter the destination lab ORI and select 'add'.
 - a. Note: the source lab and destination lab ORI will be the same and will be the ORI of the lab that generated the profile being imported into the CODIS software.
 - b. You can now delete the "srclab" source lab and "destlab" destination lab IDs in CODIS Export Manager.
- 4.) Click "OK".
 - a. The added lab IDs will stay with GMIDx from this point forward.

PREPARATION FOR CREATING A CODIS EXPORT FILE

- 1.) Prior to attempting to create a CODIS export file, complete any needed GMIDx artifact edits to the profile(s) of interest.
- 2.) It is recommended that you now save a "copy" of your GMIDx project, adding the designation "CODIS" to the name. The "CODIS" designated project will be a temporary project used only for exporting profiles to CODIS and will not be retained.
- 3.) Make any additional allele edits to the profile(s) of interest working from your "CODIS" designated project. This may include deleting peaks not intended for CODIS export or adding allele labels to stutter peaks that should be exported.
- 4.) From the GMIDx Samples tab, select the "CODIS Export" table setting (if you do not have the CODIS Export table setting option, you can import it from the GMIDx Database Defaults Table Settings folder using the GMIDx Manager window).
- 5.) For the profile(s) of interest, choose the correct Specimen Category from the drop-down (e.g. "Forensic, Unknown", "Forensic Mixture", "Suspect"). The specimen category can be updated if needed once the profile is imported into CODIS.
- 6.) Leave the Specimen Category as "No Export" for any samples not to be included in the CODIS export file. If exporting profiles for STR Data Entry, multiple profiles can be exported in one CODIS export file. If exporting profiles for Popstats Calculations, each profile needs to be exported into a separate CODIS export file.
- 7.) Optional: type the sample name, as it is desired to appear in CODIS, into each sample's corresponding UD1 column.

NOTE: No brackets or parentheses may be present on peaks to be exported. GMIDx will not allow the OL designation to be exported, so if a true allele, OL's must be given an allele designation prior to creating the CODIS export file.

CREATING A CODIS EXPORT FILE

- 1.) Following completion of the "Preparation for creating a CODIS export file" steps above, from the GMIDx Samples tab, choose File > Export Table for CODIS.
- 2.) Select export file type CMF 3.2 (.xml).
- 3.) Select the appropriate source and destination CODIS laboratory IDs, if not already defaulting correctly.
- 4.) Enter the desired file name.
- 5.) Navigate to the desired export file location. e.g. Computer > Local Disk (USB Thumb Drive).
- 6.) Click "Export".

NOTE: Once a CMF file has been exported from GMIDx, it cannot be edited – CODIS will not accept the file if it has been altered after creation.

IMPORTING DATA TO CODIS – STR DATA ENTRY

- 1.) In the CODIS Software, open Specimen Manager from the Workbench Explorer.
- 2.) At the top of the window, click on the Specimen Manager drop-down and select “Import Specimens”.
 - a. Navigate to and select the desired file to be imported from your portable media storage device.
 - b. Click “Open”.
 - c. Ensure the Import Type is “Data Import” and the Assign to User field is associated with the analyst that produced the DNA profile.
 - d. Click “OK”.
 - i. A message that the file imported successfully should appear, click “OK”. The profile(s) have now been imported into the CODIS Software, but have not yet been entered as specimens in Specimen Manager.
- 3.) Open Message Center.
 - a. Double-click “Import STR Files” in the navigation pane.
 - i. The imported file should be present in bold – select by double-clicking on the file. If the imported file is not present in bold, click ‘Refresh’ in the Import STR Files drop-down at the top of the window.
 - ii. In this step, the CODIS software is validating the import file for suitability. If the validation passes, the profile(s) contained in the import file will now be entered as specimens in Specimen Manager.
 - b. Double-click “Import Reports” in the navigation pane.
 - i. The imported file report should be present in bold – select by double-clicking on the file. If the imported file report is not present in bold, click ‘Refresh’ in the Import STR Files drop-down at the top of the window.
 - ii. Two reports will now open, the LDIS Import Reconciliation Summary and Details Reports. Review both reports for accuracy and for the presence of any error codes. If any error codes are present, see your local CODIS Administrator. (Error codes will show up on the Details Report to the left of each profile’s sample name).
 - iii. Close the Reconciliation Reports after completion of review.
- 4.) Open STR Data Entry.
 - a. Query for the imported specimen(s).
 - b. Select the appropriate sample and click “OK”.
 - c. Update the Specimen ID, Specimen Category, Source ID, and Partial Profile Indicators as necessary.
 - d. Save and Print the LDIS Specimen Detail Report.

IMPORTING DATA TO CODIS – POPSTATS

- 1.) In the CODIS Software, open Popstats.
- 2.) Choose the desired Popstats Calculations option (e.g. Forensic Single Source, Forensic Mixture, Match Estimation).
- 3.) In the Target Profile window, click “Import”.
- 4.) Navigate to and select the desired file to be imported from your portable media storage device.
- 5.) Click “Open”.
- 6.) Continue with calculations according to current casework practices.
 - a. For calculations that require more than one input (e.g. Forensic Mixture: Mixture, H1, H2), GMIDx can be used to create a tailored CODIS export file for each input with allele labels added to account for stutter or peaks deleted as needed.

STR CASE FILE CONTENT

A case file will contain, at minimum, the following items filled out with sufficient information as to appropriately record all of the data pertinent to the particular case. Additional copies of some items may be necessary for appropriate documentation.

ADMINISTRATIVE DOCUMENTATION

1. RFLE form(s)
2. Laboratory report
 - a. Copies of issued reports
 - b. Copies of amended reports
3. Draft of laboratory report showing documentation of the technical peer review
4. Chain of custody is part of case record and is held electronically in LIMS
5. Cover letters, officer's reports, other information relevant to the case
6. CD containing relevant electronic data
 - a. GeneMapper® ID projects and run folder(s)
 - b. Size Standards, and Analysis Method(s)
 - c. Scans and digital photos (when appropriate)
 - d. Quantification file(s)

EVIDENCE EXAMINATION AND STR ANALYTICAL DOCUMENTATION

1. Evidence examination documentation including photos
2. Communication(s) authorizing consumption of DNA evidence (when appropriate)
3. Appropriate Worksheet(s) found on the FLSB Portal to document as appropriate:
 - a. Extraction
 - b. Quantitation
 - c. Clean-up/purification
 - d. Amplification
 - e. CE detection, sample location, and analysis review
 - f. Sample analysis
4. Population Frequency data sheets (when appropriate)
5. CODIS-related data sheets

STRMIX™ CASE FILE CONTENTS

1. All files in the each Results folder (see Single Sample Analysis step 9) will be included in the electronic data associated with the case file.
2. The pages of the final Deconvolution Report up to, and including, the "Evidence Input File" will be printed and included in the case file. If multiple deconvolutions were performed, all should be included in the electronic data, but on the final one used for the report needs to be printed.

3. If a LR is calculated the pages of the “LR From Previous Report” up to, and including, the LR for all racial groups will be printed and included in the case file. Any other calculated LRs will be included in the electronic data, but will not be printed.
 - a. Exclusionary LRs will be included in the electronic data, but will not be printed.
4. Reference profiles may be printed from the “LR From Previous Report” (“Reference Files” section) if applicable, or they may be printed on Form 7223 – Reference Samples F6C.
5. Manual simple exclusions shall be documented on either the printed pages of the Deconvolution Report or Form 7223 where the reference profile is documented.

STR CASE FILE REVIEW

Case file reviews will be performed on each case file and will conform to the policy and procedures outlined in the CLD Quality/Operations Manual. Case file technical review will encompass all of the standard case file considerations in addition to the following, which are specific to the evaluation of the electronic data generated during STR analysis. Each of the electronic data files used to support a conclusion is evaluated to ensure that the criteria outlined within the Guidelines sections (Evaluating DNA Typing Profile Data, Interpretation of DNA Typing Profiles, Statistical Interpretation of STR DNA Typing Profiles, and Report Writing) contained within this manual have been met.

PERFORMING PEER/TECHNICAL REVIEW OF GENEMAPPER® *ID-X* ELECTRONIC DATA

The manual edits made by the analyst during evaluation of the data can be examined by the reviewer using the tools and features within the GeneMapper® *ID-X* software.

View Samples with Manual Edits and Overrides

1. Open the GeneMapper® *ID-X* project.
2. In the Project window, select **View CGQ Overrides** from the Table Setting drop-down list. Sample(s) displayed in this list were manually accepted by the analyst with or without edits.
3. In the Project window, select View Edited Samples from the Table Setting drop-down list. Sample(s) with at least one allele or artifact label edit are displayed.
4. Verify the peak edits.

View Edits in the Label Edit Viewer

The Label Edit Viewer contains a list of edits made to the allele and artifact labels displayed in the sample electropherogram plots of the Samples plot. View the Label Edit Viewer from the Project window or the Samples plot.

1. In the Project window, make sure **View Edited Samples** is selected from the Table Setting drop-down list.
2. Select the edited sample in the filtered Samples table and select to **Display Plots**.
3. In the Samples plot, select the View Label Edits setting from the Plot Setting drop-down list to display the plots above the list of edits in the Label Edit Viewer table.
4. Verify the peak edits. Select a row in the Label Edit Viewer table and the corresponding edited peak will be highlighted.

View Edits from the Project Window

1. In the Project window, make sure **View Edited Samples** is selected from the Table Setting drop-down list.
2. Select the edited sample in the filtered Samples table and select to open the **Label Edit Viewer** in a separate window.

3. If desired, export and print the Label Edit Viewer data. Close the Label Edit Viewer table to return to the filtered Samples table.
4. Verify the peak edits.

View Allele Edits and Comments in the Genotypes Table and Genotypes Plot

Genotypes Table

1. In the Project window, select the **Project** node in the navigation pane then select the **Genotypes** tab.
2. Make sure the **View Edited Samples** setting is selected from the Table Setting drop-down list.
3. Review the entries in the following table columns:
 - a. **Allele Edit (AE) Reason for Change:** Displays the last reason for change entered for an edit that yields an allele label.
 - b. **Marker Edit Comment (MEC):** Displays the reason for change entered for an edit that yields an artifact label or when alleles are deleted.
 - c. **Marker Edit (ME) flag:** Displays a green check mark (true) if allele or artifact labels are edited within a marker size range.
4. Verify the peak edits.

Genotypes Plot

1. In the Project window, Shift-click to select all rows in the Genotypes table then select to **Display Plots**.
2. In the Genotypes plot, select the **Traditional Genotype Plot** setting from the Plot setting drop-down list. This plot setting displays one marker per pane for each of the markers selected in the Genotypes table.
3. Select **File** and **Close Plot Window** to return to the Project window.
4. Verify the peak edits.

OVERALL REVIEW OF ELECTRONIC DATA

Only sample files pertaining to the case in review shall be found in the case file run folders.

1. Ensure all of the amplified samples are represented on the GMID Sample Location sheet.
2. Ensure all of the listed samples on the GMID Sample Location sheet are referenced to their appropriate run folder and are present within the GeneMapper project.
3. Ensure that the appropriate Analysis Parameters used for analysis are saved electronically with the case file.
4. Examine each sample file saved in the GeneMapper® project as appropriate (i.e. raw data, size standard examination, injection duration).

5. Ensure the appropriate sample analysis worksheets (i.e. single source or mixture sheets) are present and have been filled out.
 - a. An independent analysis of the evidence sample(s) shall be performed. The reviewer should be in agreement with the statements about the number of possible contributors, major and minor components, etc.
6. Where applicable, the appropriate statistics report(s) is present. If a random match probability is reported, verify that the appropriate formula was used.
7. When appropriate, the appropriate CODIS search/match documentation is present.
8. The allele table(s) in the case file (if present) will be compared to the GeneMapper® results to ensure consistency.
9. **Verbal reports will conform to the policy and procedures outlined in the CLD Quality Manual section 9.5.2.** If permission is obtained to issue a verbal DNA interpretation report, it must be peer reviewed prior to release. This would include GeneMapper® results for all appropriate samples and controls. If a statistical interpretation is to be included in the verbal report, the Popstats data must also be reviewed. The written verbal report will be maintained in the case file and used to check for consistency when the formal written report is reviewed. A technical peer review is not necessary for verbal communications to the investigator of non-comparative results (such as body fluid identification). This communication shall be documented in the case file notes.
10. The case file shall contain an approved Technical Review Worksheet.

STRMIX™ TECHNICAL REVIEW

1. Input information documented on the Deconvolution Report shall be checked against GeneMapper® ID-X.
2. Check correct pages of the Deconvolution Report have been printed.
3. Check and evaluate the log(likelihood) value, Gelman-Rubin convergence diagnostic value, and the Variance Charts.
4. Check the Summary of Contributors makes intuitive sense.
5. If a profile was entered into CODIS based on STRmix™ genotype weights, check that the entered genotypes make intuitive sense, are supported by the STRmix™ data, and that all genotype options that add up to ≥99.00% are accounted for.
6. If a LR was calculated, ensure the correct pages of the “LR From Previous Report” are printed in the case file.
7. Ensure all necessary files are included in the electronic data.

OWNERSHIP REVIEW OF CASEWORK SAMPLES CONTRACTED WITH OUTSIDE VENDOR LABORATORIES FOR CODIS ELIGIBILITY

The ownership review of outsourced samples being submitted for CODIS entry will encompass standard WSP case file considerations and will be focused on the sample(s) under consideration for CODIS eligibility. In addition, all of the criteria for data evaluation outlined in the technical specifications section of the Service Agreement that are put in place for the specific vendor will be evaluated. A vendor specific ownership review form will be filled out by the reviewing scientist and retained in the case file. The electronic data of a DNA profile deemed eligible for CODIS upload shall be re-analyzed (either by using the vendor created GMID-X project [Sorenson Forensics Fusion 6C data only] or by creating a new project using vendor provided data files) and results retained. Vendor specific Service Agreements are found in the Outsourcing Documents folder on the FLSB Portal. The vendor specific Ownership Review forms are found in the Forms and Templates folder on the FLSB Portal.

*Ownership Review of Casework for samples contracted with **Sorenson Forensics LLC**.*

Sorenson cases are initially accessed via download from a 'Customer Portal' at Sorenson. A casefile will contain a scanned copy of the entire case file and report and a folder containing the electrophoresis data . The outsource coordinator (or designee) will normally download, save and make cases available for reviewers to access from a local drive. Sorenson case batches may share extraction, amplification, and analysis controls. Controls may be reviewed once and then accepted for the entire batch. Ownership Review of CODIS eligible samples will include evaluation of the criteria below, which are specific to the amplification kit used to generate the data and to the Scope of Work (Exhibit A) section of the Biological Screening & DNA Testing Services Agreement with the appropriate Sorenson SOPs. The Ownership Review Checklist (Fusion 6C) will be completed by the ownership reviewer, signed, and retained in each file. Sorenson cases that are deemed to be ineligible (no data will be eligible for CODIS upload) or serology negative do not require ownership review. Ineligible cases will only undergo ownership review if any data or statistical revisions are being requested from Sorenson. For ownership review of Identifiler® Plus outsourced data, the analyst will refer to legacy protocols in Revision 30 of the WSP Casework STR Analysis Procedures to find information and interpretation guidelines.

Fusion 6C

Examine the case file documentation (.pdf files) to verify that all appropriate records and the run folders for the review to be completed are present. The ownership review should address the following points:

1. Verify the case has been entered correctly in LIMS.
2. Verify that the correct sample results for the CODIS sample are stated in the report.
3. Verify the CODIS sample packaging, description, and sampling is documented in the laboratory notes. Verify the CODIS sample can be tracked through Accessioning, Itemization, Serology, Cutting to DNA, Extraction, Quantification, Normalization, Amplification, and Analyzer sections of the laboratory notes.
4. Verify sample remains for reanalysis. If not, verify that authorization to consume is present in case file.
5. Either the Sorenson Forensics provided .ser project file may be used to reanalyze the CODIS sample, or a new .ser project file may be created to reanalyze the CODIS sample.

Review of outsourced data using the Sorenson Forensics provided .ser project file

6. Sorenson Forensics specific Analysis Methods, Panels, Bins, and Stutter files have

been created for use in GMID-X. These must be imported for use prior to analysis of any Sorenson Forensics Fusion 6C data.

7. Import the Sorenson Forensics .ser file into GMID-X. This project contains all question and reference samples for the case and allelic ladders used. The yellow status arrow will appear in GMID-X when the project is viewed. Change the “Panel” to the appropriate Sorenson Forensics specific panel. Do not choose “analyze” in GMID-X, as this will erase any Sorenson edits for the project. Confirm that the allelic ladders passed.
8. View the CODIS sample and any additional profiles used in the deduction of the CODIS sample profile (e.g. non-sperm fraction profile or reference profile used to deduce profile from the sperm fraction). Verify that the analyst agrees with all allele calls and that the allele calls match those reported in the Sorenson Forensics allele table.
9. Use the electropherograms in the Sorenson Forensics provided .pdf file to verify the reagent blank and positive and negative amplification controls associated with the CODIS sample all passed (may be done by a single analyst for shared controls in a batch).

Review of outsourced data using a newly created .ser project file

10. The electronic data associated with the CODIS sample and allelic ladder(s) shall be reanalyzed using Sorenson’s analysis parameters and retained in the case file.
 - a. Use the Sorenson Forensics specific Analysis Methods, Panels, Bins, and Stutter files that have been created for use in GMID-X.
 - b. If an additional profile is used in the deduction of the CODIS profile (e.g. non-sperm fraction profile or reference profile used to deduce profile from the sperm fraction), the electronic data from the additional profile will also be reanalyzed and retained in the case file.
11. Verify that the analyst agrees with all allele calls of the reanalyzed samples and that the allele calls match those reported in the Sorenson Forensics allele table. Verify that the allelic ladders passed.
12. Use the electropherograms in the Sorenson Forensics provided .pdf file to verify the reagent blank and positive and negative amplification controls associated with the CODIS sample all passed (may be done by a single analyst for shared controls in a batch). Alternately, the reagent blank and/or controls may be included in the .ser project file for reanalysis and verification.

Both reanalysis methods continue with the steps below

13. Verify no contamination was detected in the samples or controls. If there was a contamination or processing error in the case, it should be addressed and a log/report describing the incident included in the case file documentation.
14. Verify that the Forensic Analysis Technical/ Administrative Review Form is present, complete, and signed.
15. When verifying the allele calls of the CODIS sample:
 - a. For single source profiles, as designated by Sorenson Forensics, the Sorenson Forensics allele table can be used. Y-STR loci may be added to this table as applicable.
 - b. For mixture profiles with a clear CODIS uploadable major profile (defined as meeting the Sorenson Forensics major/minor disparity percentages at all loci

- AND no ambiguity at any locus) or a clear deduction profile with no locus ambiguity, the appropriate Sorenson Forensics allele table can be used.
- c. For all other mixture profiles, the appropriate Mixture Analysis form shall be used.
16. If additional alleles are deemed CODIS eligible, this will be documented in the case file and technically reviewed by a second qualified analyst prior to CODIS upload. Y-STR loci may be added.
 - a. If additional alleles deemed CODIS eligible are not called as alleles in the Sorenson Forensics provided .ser project file (i.e. an allele was deleted and called as an artifact), reanalysis must be conducted by a newly created .ser project file.
 - b. Profiles that require additional interpretation shall be documented on the appropriate Sample or Mixture Analysis form with the additional interpretation supported by appropriate reasoning.
 17. Verify that the CODIS sample profile meets CODIS eligibility requirements and select the appropriate specimen category and upload level following WSP protocols for sample entry. Sorenson Forensics outsourcing projects should not be used for creation of CODIS export files at this time.
 18. The DNA F6C Ownership Review Checklist will be used to document the ownership review of the Sorenson Forensics CODIS sample and related data, the CODIS sample entry, and the final case technical and administrative review steps.
 19. A CD will be created that contains the following electronic data:
 - a. Sorenson Forensics provided documentation and electronic files (PDF file, report file, .ser project, and data file with all run folders).
 - b. WSP .ser project (if created)
 - c. Sorenson Forensics GMID-X analysis parameters

STATISTICAL INTERPRETATION OF STR DNA TYPING

The strength of DNA evidence may be assessed by different statistical methods: random match probability and likelihood ratio calculation as stated in the Scientific Working Group on DNA Analysis Methods Interpretation Guidelines for autosomal STR typing by forensic DNA laboratories (SWGDAM 2010). The approach taken depends on the forensic question to be addressed, the DNA typing analysis data available and the practicality of the approach. Calculations of genotype frequencies are explained in the recommendation report from the National Research Council Committee on DNA Forensic Science (1996). The FBI population databases as published by Budowle et al. in the *Journal of Forensic Sciences* (1999, 2001, 2015), FSI Genetics (2016), and in *Forensic Science Communications* (2001) and found in CODIS Popstats are utilized. A minimum allele frequency estimation is employed for STR loci which demonstrate alleles with a low frequency of occurrence. The formula used to calculate the minimum allele frequency is $5/2N$, where N is the number of individuals in the database.

SINGLE SOURCES (OR UNAMBIGUOUS SINGLE PROFILES IN MIXED ORIGIN SAMPLES)

For heterozygous genotypes, the genotype frequency is twice the product of the frequency of occurrence of one allele and the frequency of occurrence of the other allele:

$$2pq$$

For homozygous genotypes, the genotype frequency is calculated as:

$$p^2 + p(1-p) \theta$$

where p is the allele frequency and theta (θ) is to compensate for population structure. A θ value of 0.01 will be generally used, with a θ value of 0.03 reserved for smaller, isolated populations (such as Native American).

If only 1 allele is detected at a locus and is at risk of not having a sister allele detected due to stochastic effects from being at a low level then following 5.2.1.3.1 from SWGDAM 2010 and 4.1 of NRCII the formula becomes:

$$2p$$

To avoid double counting the proportion of homozygotes in the population the formula becomes:

$$2p - p^2$$

Both can be used in the Popstats program. The 5.2.1.3.2 SWGDAM 2010 algebraically identical formula that can be used is:

$$p^2 + 2p(1-p)$$

To account for population substructure the formula then becomes:

$$p^2 + p(1-p) \theta + 2p(1-p)$$

The genotype frequency across several loci is calculated by multiplying the frequencies of the individual STR locus frequencies (Product Rule). Results reported as a frequency of occurrence (or Random Match Probability) will be expressed conservatively by truncating to the second significant digit. For example, 1 in 194,584 would be reported as 1 in 190,000; 1 in 456,397,565 would be reported as 1 in 450 million; and 1 in 22,199,677,375 would be reported as 1 in 22 billion.

If possible contributors of the DNA typing evidence involve relatives, their DNA profiles should be obtained for exclusionary purposes. If these DNA profiles cannot be obtained, conditional probabilities can be calculated following the formulae 4.8a, 4.8b, 4.9a, or 4.9b from the National Research Council Committee on DNA Forensic Science (1996) report

MIXTURES (NO DISTINGUISHABLE MAJOR PROFILE)

Following 5.4 from SWGDAM 2010, the strength of DNA evidence for mixtures can be assessed by using a likelihood ratio (LR) approach. This would involve a situation with two competing hypotheses regarding the origin of the evidence. The calculation of the LR in a mixture is dependent upon the evidence profile, the comparison reference profile(s), and the individual hypotheses. The databases and calculations based on Weir et al 1997 which are in the FBI POPSTATS program can be utilized for this purpose.

Formula for calculating Likelihood Ratio

$$LR = \text{Prob (Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d)$$

The unrestricted combinatorial approach for the scenario of a 2 person mixture with one known (victim) is the simplest application and is illustrated as follows.

4 allele scenario

Evidence	Victim	Suspect
— A ₁		— A ₁
— A ₂		— A ₂
— A ₃	— A ₃	
— A ₄	— A ₄	

victim reference is A₃, A₄ and suspect reference is A₁, A₂

$$LR = \text{Prob (Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d) = 1/2p_12p_2$$

3 allele scenario 1

Evidence	Victim	Suspect
— A ₁		— A ₁
— A ₂		— A ₂
— A ₃	— A ₃	

victim reference is A₃, A₃ and suspect reference is A₁, A₂

$$LR = \text{Prob(Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d) = 1/2p_12p_2$$

3 allele scenario 2

Evidence	Victim	Suspect
— A ₁		— A ₁
— A ₂	— A ₂	

— A₃ — A₃

victim reference is A₂, A₃ and suspect reference is A₁, A₁

$$LR = \text{Prob (Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d) = 1/(p_1^2 + 2p_1p_2 + 2p_12p_3)$$

Other scenarios follow below

Evidence	Victim	Suspect	H _p	H _d
A ₁ , A ₁	A ₁ , A ₁	A ₁ , A ₁	1	p_1^2
A ₁ , A ₂	A ₁ , A ₂	A ₁ , A ₂	1	$p_1^2 + 2p_1p_2 + p_2^2$
A ₁ , A ₂	A ₁ , A ₁	A ₂ , A ₂	1	$2p_1p_2 + p_2^2$

The scenario of a 2 person mixture with a suspect match and an unknown contributor is as follows.

4 allele scenario

Evidence	Suspect
— A ₁	— A ₁
— A ₂	— A ₂
— A ₃	
— A ₄	

suspect reference is A₁, A₂

The H_p is suspect = A₁, A₂ and unknown contributor = A₃, A₄

or $2p_3p_4$

The H_d is both are unknown contributors and the possibilities are:

<u>Unk 1</u>	<u>Unk 2</u>	
A ₁ A ₂	A ₃ A ₄	$2p_1p_2 \times 2p_3p_4$
A ₁ A ₃	A ₂ A ₄	$2p_1p_3 \times 2p_2p_4$
A ₁ A ₄	A ₂ A ₃	$2p_1p_4 \times 2p_2p_3$
A ₃ A ₄	A ₁ A ₂	$2p_3p_4 \times 2p_1p_2$
A ₂ A ₄	A ₁ A ₃	$2p_2p_4 \times 2p_1p_3$
A ₂ A ₃	A ₁ A ₄	$2p_2p_3 \times 2p_1p_4$
		□□□□□□□□
		$24 p_1p_2p_3p_4$

$$LR = \text{Prob (Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d) = 1/12 p_1p_2$$

3 allele scenario

Evidence	Suspect
— A ₁	— A ₁
— A ₂	— A ₂
— A ₃	

suspect reference is A₁, A₂ and an unknown contributor is at least an A₃

The H_p is suspect = A₁, A₂, and unknown contributor is the 3 possible combinations of A₃ with itself and A₁, A₂

or $p_3^2 + 2p_1p_3 + 2p_2p_3$

The H_d is all 12 possible genotype combinations from unknown contributors

or $12 (p_1p_2p_3) (p_1+p_2+p_3)$

$LR = \text{Prob (Evidence} \mid H_p) / \text{Prob (Evidence} \mid H_d) =$

$[p_3^2 + 2p_1p_3 + 2p_2p_3] / [12 (p_1p_2p_3) (p_1+p_2+p_3)]$

or $[p_3+2p_1+2p_2] / [12 p_1p_2 (p_1+p_2+p_3)]$

The consideration of peak height and ratios to eliminate possible genotype combinations which could not occur for the assumed number of contributors is considered a restricted LR calculation.

LIKELIHOOD RATIOS FOR PATERNITY AND PARENTAGE RELATEDNESS

STR DNA profiling has proven to be a preferred method of DNA testing for parentage testing including the standard paternity testing trio of mother, child and alleged father. According to the American Association of Blood Banks AABB Relationship Testing Program Unit 2008 Annual Report for Testing over 99% of all testing involved STRs. The standard calculation to statistically assess paternity STR result comparisons is the combined paternity index which is a likelihood ratio calculation.

Paternity

Prob(E/M,C,F) - is the probability that the evidence would be observed given that the mother and alleged father were the biological parents of the child.

Prob(E/M,C) - is the probability that the evidence would be observed given that a random member of the population was the biological father.

LR - is the likelihood ratio of the two probabilities.

Table 1 - Paternity Calculations in cases where mother's, child's and alleged father's genotypes are known

	Mother	Child	Alleged Father	Prob(E M,C,F)	Prob(E M,C)	LR
1	AA	AA	AA	$P_A^2 \times P_A^2$	$P_A^2 \times P_A^2 \times P_A$	$1/P_A$
2	AA	AA	AB	$P_A^2 \times 2 P_A P_B \times \frac{1}{2}$	$P_A^2 \times 2 P_A P_B \times P_A$	$1/(2P_A)$
3	AA	AB	BB	$P_A^2 \times P_B^2$	$P_A^2 \times P_B^2 \times P_B$	$1/P_B$
4	AA	AB	AB	$P_A^2 \times 2 P_A P_B \times \frac{1}{2}$	$P_A^2 \times 2 P_A P_B \times P_B$	$1/(2P_B)$
5	AA	AB	BC	$P_A^2 \times 2 P_B P_C \times \frac{1}{2}$	$P_A^2 \times 2 P_B P_C \times P_B$	$1/(2P_B)$
6	AB	AB	BB	$2 P_A P_B \times P_B^2 \times \frac{1}{2}$	$2 P_A P_B \times P_B^2 \times \frac{1}{2} \times (P_A + P_B)$	$1/(P_A + P_B)$
7	AB	AB	AB	$2 P_A P_B \times 2 P_A P_B \times (\frac{1}{4} + \frac{1}{4})$	$2 P_A P_B \times 2 P_A P_B \times \frac{1}{2} \times (P_A + P_B)$	$1/(P_A + P_B)$
8	AB	AB	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times (P_A + P_B)$	$1/(2P_A + 2P_B)$
9	AB	AA	AA	$2 P_A P_B \times P_A^2 \times \frac{1}{2}$	$2 P_A P_B \times P_A^2 \times \frac{1}{2} \times P_A$	$1/P_A$
10	AB	AA	AB	$2 P_A P_B \times 2 P_A P_B \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_B \times \frac{1}{2} \times P_A$	$1/(2P_A)$
11	AB	AA	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times P_A$	$1/(2P_A)$
12	AB	AC	CC	$2 P_A P_B \times P_C^2 \times \frac{1}{2}$	$2 P_A P_B \times P_C^2 \times \frac{1}{2} \times P_C$	$1/P_C$
13	AB	AC	BC	$2 P_A P_B \times 2 P_B P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_B P_C \times \frac{1}{2} \times P_C$	$1/(2P_C)$
14	AB	AC	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times P_C$	$1/(2P_C)$
15	AB	AC	CD	$2 P_A P_B \times 2 P_C P_D \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_C P_D \times \frac{1}{2} \times P_C$	$1/(2P_C)$

It is generally accepted that more than two loci must demonstrate an exclusion (mutation is proposed as the reason for the exclusion) before the evidence is indicative of a paternal exclusion. The LR accounting for the possibility of mutation at any specific locus is calculated as μ/\bar{A} where μ is the locus specific mutation rate and \bar{A} is the mean power of exclusion. See the table at the end of this section for locus specific mutation rate and power of exclusion.

The likelihood ratio can also be used to assess STR result comparisons for identifying missing individuals.

Parentage (missing child)

Prob(E/M,C,F) - is the probability that the evidence would be observed given that the alleged child is/was a biological child of the mother and father.

Prob(E/M,C) - is the probability that the evidence would be observed given that the alleged child is/was a random member of the population.

LR - is the likelihood ratio of the two probabilities.

Table 2 - Parentage Calculations in missing child cases where mother's and father's genotypes are known

	Mother	Q	Father	Prob(E M, F, Q)	Prob(E M, F, U)	LR
1	AA	AA	AA	$P_A^2 \times P_A^2$	$P_A^2 \times P_A^2 \times P_A^2$	$1/P_A^2$
2	AA	AA	AB	$P_A^2 \times 2 P_A P_B \times \frac{1}{2}$	$P_A^2 \times 2 P_A P_B \times P_A^2$	$1/(2P_A^2)$
3	AA	AB	BB	$P_A^2 \times P_B^2$	$P_A^2 \times P_B^2 \times 2P_A P_B$	$1/(2P_A P_B)$
4	AA	AB	AB	$P_A^2 \times 2 P_A P_B \times \frac{1}{2}$	$P_A^2 \times 2 P_A P_B \times 2P_A P_B$	$1/(4P_A P_B)$
5	AA	AB	BC	$P_A^2 \times 2 P_B P_C \times \frac{1}{2}$	$P_A^2 \times 2 P_B P_C \times 2P_A P_B$	$1/(4P_A P_B)$
6	AB	AB	BB	$2 P_A P_B \times P_B^2 \times \frac{1}{2}$	$2 P_A P_B \times P_B^2 \times 2P_A P_B$	$1/(4P_A P_B)$
7	AB	AB	AB	$2 P_A P_B \times 2 P_A P_B \times (\frac{1}{4} + \frac{1}{4})$	$2P_A P_B \times 2P_A P_B \times 2P_A P_B$	$1/(4P_A P_B)$
8	AB	AB	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2P_A P_B \times 2P_A P_C \times 2P_A P_B$	$1/(8P_A P_B)$
9	AB	AA	AA	$2 P_A P_B \times P_A^2 \times \frac{1}{2}$	$2P_A P_B \times P_A^2 \times P_A^2$	$1/(2P_A^2)$
10	AB	AA	AB	$2 P_A P_B \times 2 P_A P_B \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_B \times P_A^2$	$1/(4P_A^2)$
11	AB	AA	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C \times P_A^2$	$1/(4P_A^2)$
12	AB	AC	CC	$2 P_A P_B \times P_C^2 \times \frac{1}{2}$	$2 P_A P_B \times P_C^2 \times 2P_A P_C$	$1/(4P_A P_C)$
13	AB	AC	BC	$2 P_A P_B \times 2 P_B P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_B P_C \times 2P_A P_C$	$1/(8P_A P_C)$
14	AB	AC	AC	$2 P_A P_B \times 2 P_A P_C \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C \times 2P_A P_C$	$1/(8P_A P_C)$
15	AB	AC	CD	$2 P_A P_B \times 2 P_C P_D \times \frac{1}{2} \times \frac{1}{2}$	$2 P_A P_B \times 2 P_C P_D \times 2P_A P_C$	$1/(8P_A P_C)$

Most calculations encountered by the crime lab can be done using the FBI POPSTATS program. However occasionally more specialized calculations are required and manual calculations can be documented in case notes.

Single Parent Calculations - father

Prob(E | U,C,F) - is the probability that the evidence would be observed given that the alleged father was the biological parent of the child.

Prob(E | U,C,U) - is the probability that the evidence would be observed given that the alleged father was not the biological father of the child.

LR - is the likelihood ratio of the two probabilities.

Table 3 - Paternity Calculations in cases where the child's and alleged father's genotypes are known

	Child	Alleged Father	Prob(E U,C,F)	Prob(E U,C, U)	LR
1	AA	AA	$P_A^2 \times P_A^2$	$P_A^2 \times P_A^2$	$1/P_A$
2	AA	AB	$P_A \times 2 P_A P_B \times \frac{1}{2}$	$P_A^2 \times 2 P_A P_B$	$1/(2P_A)$
3	AB	AA	$P_B \times P_A^2$	$2 P_A P_B \times P_A^2$	$1/(2P_A)$
4	AB	AB	$(P_A + P_B) \times 2 P_A P_B \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_B$	$(P_A + P_B) / (4P_A P_B)$
5	AB	AC	$P_A^2 \times 2 P_B P_C \times \frac{1}{2}$	$2 P_A P_B \times 2 P_A P_C$	$1/(4P_A)$

For scenarios not covered contact the DNA Technical Lead for assistance.

Likelihood Ratios for Kinship

Kinship evaluates the likelihood that the pair of given DNA profiles are associated by kinship vs. by chance. More meaningful statistics can be calculated for full profiles and more family members. Given a pair of matching (high, moderate or low stringency) DNA profiles, the likelihood ratio of the two DNA profiles being from relatives vs. the two DNA profiles being unrelated is calculated for each locus as follows:

Relationship	Notation	ϕ_2	ϕ_1	ϕ_0
Self (MZ twin)	MZ	1	0	0
Parent-Offspring	PO	0	1	0
Full Sib	FS	1/4	1/2	1/4
Half-Sib ¹ , Uncle/Aunt- Nephew/Niece, Grandparent- Grandchild	HS	0	1/2	1/2
Half-Sib whose parents are sibs	HS-S	1/8	1/2	3/8
Half-Sib whose parents are half- sibs	HS-HS	1/16	1/2	7/16
First cousin	1C	0	1/4	3/4
Double first cousin	D	1/16	3/8	9/16
Unrelated	U	0	0	1

Where ϕ_0 is the probability that the locus genotypes of the two individuals share no allele by descent for the specified kinship; ϕ_1 is the probability that the locus genotypes of the two individuals share one allele by descent for the specified kinship; ϕ_2 is the probability that the locus genotypes of the two individuals share two alleles by descent for the specified kinship.

#	G_x (Ref)	G_y (E)	Conditional Probability of G_y given G_x under		
			I (2 alleles IBD)	T (1 allele IBD)	O (0 allele IBD)
			$P_2(X,Y)$	$P_1(X,Y)$	$P_0(X,Y)$
1	A	A	1	a	a^2
2	A	B	0	0	b^2
3	A	AB	0	b	$2ab$
4	A	BC	0	0	$2bc$
5	AB	A	0	$\frac{a}{2}$	a^2
	AB	B		$\frac{b}{2}$	b^2
6	AB	C	0	0	c^2
7	AB	AB	1	$\frac{a+b}{2}$	$2ab$
8	AB	AC	0	$\frac{c}{2}$	$2ac$
	AB	BC		$\frac{c}{2}$	$2bc$
9	AB	CD	0	0	$2cd$

where:

$P_2(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 2 of their alleles identical by descent (IBD);

$P_1(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 1 of their alleles identical by descent (IBD);

$P_0(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 0 of their alleles identical by descent (IBD).

The probability of observing the locus genotype G_y (reference) given G_x (evidence) for a specified kinship is:

$$P(\text{kinship}) = [P_2(X,Y) \times \phi_2] + [P_1(X,Y) \times \phi_1] + [P_0(X,Y) \times \phi_0]$$

The likelihood of the locus genotype G_y (reference) given G_x (evidence) being related by specified kinship vs. the locus genotypes being unrelated is:

$$LR(\text{kinship}) = \frac{P(\text{kinship})}{P(\text{unrelated})}$$

To summarize, for each locus:

	Kinship LR	Kinship Probability
Locus Formula	$\frac{P(\text{kinship})}{P(\text{unrelated})}$	$P(\text{kinship})$

Combined probability for the specified kinship is:

$$CP(\text{kinship}) = \prod_{\text{locus}} P(\text{kinship})$$

And the combined likelihood ratio for the profiles to be related by the specified kinship vs unrelated is:

$$CLR(\text{kinship}) = \prod_{\text{locus}} LR(\text{kinship})$$

Single Parent Kinship Calculation

In the special case where the relationship is parent-child (PO), the calculation of the generalized PI stated in the General Kinship Formula topic can be simplified. This is the so-called “single parent” case, or the “motherless paternity” case. In addition to the parentage index, the probability of parentage and the probability of exclusion can be derived for this special case. More meaningful results can be obtained when both parents are tested. However in the case where only one parent and the child can be obtained the following calculations can be done. The locus genotype G_y comes from the child, and the locus genotype G_x comes from the alleged parent. Using the tables in the General Kinship Formula topic, the formulae for the parentage index simplify to:

	Child (C)	Alleged Parent (AP)	Parentage Index (PI)	Probability of Exclusion (PE)⁵
1.	A	A	$\frac{1}{a}$	$(1 - a)^2$
2.	A	AB	$\frac{1}{2a}$	$(1 - a)^2$
3.	AB	AB	$\frac{a + b}{4ab}$	$[1 - (a + b)]^2$
4.	AB	A	$\frac{1}{2a}$	$[1 - (a + b)]^2$
5.	AB	AC	$\frac{1}{4a}$	$[1 - (a + b)]^2$

In a case where an alleged parent has an inconsistent genotype at one locus, that is, the genotype of the alleged parent and the genotype of the child do not share any allele, then:

	Child (C)	Alleged Parent (AP)	Parentage Index (PI)	Probability of Exclusion (PE) ⁶
1.	A	B	$\frac{\mu}{\bar{A}}$	$(1 - a)^2$
2.	A	BC	$\frac{\mu}{\bar{A}}$	$(1 - a)^2$
3.	AB	CD	$\frac{\mu}{\bar{A}}$	$[1 - (a + b)]^2$
4.	AB	C	$\frac{\mu}{\bar{A}}$	$[1 - (a + b)]^2$

Where μ is the mutation rate and \bar{A} is the mean power of exclusion.

Locus specific mutation rates and mean power of exclusion values to be used are the default values in POPSTATS which are the American Association of Blood Banks (AABB) values from 2003 Annual report and are provided by NIST.

The probability of parentage (W) for each locus is:

$$W = \frac{PI \cdot (\text{prior probability})}{PI \cdot (\text{prior probability}) + [1 - (\text{prior probability})]}$$

The combined parentage index (CPI) is:

$$CPI = \prod_{\text{locus}} PI.$$

The combined probability of parentage (CW) is:

$$CW = \prod_{\text{locus}} W.$$

The combined probability of exclusion (CPE) is:

$$CPE = 1 - \prod_{\text{locus}} (1 - PE).$$

Note: Rounding rules and significant figures used in POPSTATS for STR locus genotype calculation are also used in the subsequent DNA profile calculations (e.g. the Product Rule, Parentage and Kinship) however POPSTATS keeps one extra digit in memory for all of the locus genotype calculations to prevent extra error due to rounding.

For the Probability of Paternity, Maternity, or Parentage (W) rounding occurs one digit after the last nine (e.g. if 99.9992345% is the value of a PE, then with 4 meaningful decimal places it should be reported as 99.9992%) and if there is any W with more than 12 nines, it is truncated after the 12th nine and designated with a greater-than sign (e.g. >99.9999999999%).

Y-STR CASEWORK

QUALITY ASSURANCE & CONTROL

The following quality procedures are to be followed in addition to the quality assurance practices outlined in the current version of the DNA Analysis Quality Assurance Manual and the DNA Casework STR Analysis Procedures for autosomal STR typing.

Controls and Standards

Reagent Blank

A reagent blank that was extracted with the original sample set must also be amplified with the Yfiler® kit or Y23 kit. The reagent blank will be amplified using the same concentration and instrument conditions (load volume and injection times) as required by the sample in the extraction set containing the least amount of DNA. (See the Controls and Standards Section of the DNA Casework STR Analysis Procedures manual for exceptions regarding the use of a reagent blank.)

Amplification Controls

Each Yfiler® amplification set will include two amplification controls: the male Control DNA 007 and a negative control containing only TE buffer.

Each Y23 amplification set will include two amplification controls: the male Control 2800M and a negative control containing only amplification grade water.

Allelic Ladder

The Yfiler® allelic ladder will be included with every set of Y-STR samples run on the CE unit. This allelic ladder contains the most common alleles for each locus amplified in the kit.

The Y23 allelic ladder will be included with every set of Y23 Y-STR samples run on the CE unit. This allelic ladder contains the most common alleles for each locus amplified in the kit.

Sample Switch Detection Procedure

At least one, or a combination of more than one, sample switch detection protocols must be used if there is a need to process two or more male reference samples from the same case. Please refer to the Quality Assurance/Quality Control, Controls and Standards section of this manual for additional information.

Y-STR AMPLIFICATION

The desired amplification target for male template DNA for the Yfiler® kit is approximately 0.5-1.0 ng using Y quantification data. Lower targets of male template DNA may be amplified, with no lower limit. Amplifying quantities greater than 1ng should be performed with caution as they may result in saturated Y-STR profiles.

The desired amplification target for male template DNA for the Y23 kit is approximately 0.25 – 0.5ng using Y quantification data. Lower targets of male template DNA may be amplified, with no lower limit. Amplifying quantities greater than 1 ng should be performed with caution as they may result in saturated Y-STR profiles.

Yfiler® kit

1) Preparation of DNA Samples

Using the quantification results, calculate the volume needed for each test sample to provide the desired amount of DNA for amplification.

If required, a reduction in the volume and/or combination of DNA extracts can be performed before amplification. (See the Vacufuge™ Procedure – Concentration, Preservation, And Recovery of DNA Extracts and/or Microcon® Concentration of DNA protocols of the DNA Casework STR Analysis Procedures manual for further information.)

A TE/DNA mixture of the appropriate DNA concentration may be prepared at this step if desired. Alternatively, the TE and DNA can be added to the amplification tubes separately. The total volume of TE and DNA sample added to the PCR reaction is 10 µL.

2) Amplification Set-Up

Prepare the components from the amplification kit by vortexing and pulse spinning. (The following shall be performed in a dedicated PCR amplification hood.)

a. Preparation of PCR Master Mix:

Component	Vol. per Sample (µL)
Yfiler® PCR Reaction Mix	9.2
Yfiler® Primer Set	5.0
AmpliAq Gold® DNA Polymerase	0.8

Note: Additional sample reactions can be added into the calculation. The extra volume compensates for loss that may occur during pipette transfers.

b. Aliquot 15µL of master mix per tube.

c. Add 10µL of the TE/DNA template samples to their appropriate tubes.

d. Prepare Amplification Controls:

Add 5µL-10µL of male control DNA (0.1 ng/µL) to appropriate tube.

Add 10µL of TE buffer (used as a negative control) to the appropriate tube.

3) Load the samples on a GeneAmp® 9700 thermal cycler and start the appropriate amplification protocol.

4) Thermal cycler Parameters Yfiler®

Initial Incubation: 95°C, 11 minutes
Cycles: 1

Denaturation 94°C, 1 minute
Annealing: 61°C, 1 minute
Extending: 72°C, 1 minute
Cycles: 30

Final Extension: 60°C, 80 minutes
Hold/Soak: 4°C, Infinity

Y23 Kit

1) Preparation of DNA Samples

Using the quantification results, calculate the volume needed for each test sample to provide the desired amount of DNA for amplification.

If required, a reduction in the volume and/or combination of DNA extracts can be performed before amplification. (See the Vacufuge™ Procedure – Concentration, Preservation, And Recovery of DNA Extracts and/or Microcon® Concentration of DNA protocols of the DNA Casework STR Analysis Procedures manual for further information.)

An amplification grade water/DNA mixture of the appropriate DNA concentration may be prepared at this step if desired. Alternatively, the water and DNA can be added to the amplification tubes separately. The total volume of water and DNA sample added to the PCR reaction is 17.5 µL.

Note: If the DNA template has a TE buffer concentration greater than 15X, the volume of sample added should not exceed 5 µL.

2) Amplification Set-Up

Prepare the components from the amplification kit by vortexing for 15 seconds before each use. (The following shall be performed in a dedicated PCR amplification hood.)

e. Preparation of PCR Amplification Mix:

Component	Vol. per Reaction (µL)
Y23 5X Master Mix	5.0
Y23 10X Primer Pair Mix	2.5

Note: Additional sample reactions can be added into the calculation. The extra volume compensates for loss that may occur during pipette transfers.

f. Aliquot 7.5 µL of master mix per tube.

g. Add 17.5 µL of the water (or amplification grade water)/DNA template samples to their appropriate tubes.

h. Add the Amplification Controls:

- i. For the positive control, briefly vortex the tube of 2800M Control DNA (10ng/µl), and then dilute an aliquot to the desired DNA concentration. Add 0.5 ng of diluted 2800M Control DNA to the appropriate tube.
- ii. For the negative amplification control, pipet amplification grade water instead of template DNA into the appropriate tube.

3) Load the samples on a GeneAmp® 9700 thermal cycler and start the appropriate amplification protocol.

4) Thermal cycler Parameters

Y23

Set the GeneAmp® PCR System 9700 ramp speed to Max Mode

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 30 cycles, then:

60°C for 20 minutes

4°C soak

Y-STR AMPLIFICATION PRODUCT PREPARATION

Yfiler® kit

1. Prepare a master mix of Hi-Di™ Formamide and GeneScan® 600LIZ® internal size standard.
For example:
 - a. (# of samples + 1) x 9 µL Formamide
 - b. (# of samples + 1) x 0.3-0.5 µL LIZ 600 Size Standard

If an injection will cover wells with no samples, add formamide (or formamide+LIZ mixture) to the blank wells.

NOTE: Formamide is a teratogen. Be sure to wear gloves.

2. Vortex and spin briefly. If making a stock tube of mixed formamide and size standard, label the tube with the lot number of LIZ added along with the date and analyst's initials.
3. Dispense 9.0 µL of LIZ/Formamide mixture into all wells that are to be used of the CE plate.
4. Add 1.0 µL allelic ladder or PCR product to each well, being careful to not introduce bubbles. The standard load volume is 1.0 µL; however, samples may be loaded at 0.5, 1.0, 1.5, or 2.0 µL volumes. A notation will be made in the case file if a non-standard load volume is used.
5. Cover plate with septa.
6. Spin briefly to remove any bubbles, if necessary.
7. Denature the samples by placing in a thermal cycler or heat block set at 95°C for 3 minutes.
8. Snap-cool the samples for 3 minutes.
9. Place plate in a plate base and cover with a plate retainer, being mindful of plate alignment.

In addition to using injection times less than validated default injection times for amplicons with excessive peak heights (e.g. causing –A shoulder artifacts or saturation), a smaller amplicon volume as described above can be loaded, or a dilution in either formamide or TE up to 1:10 can be used. If any of these methods or combinations thereof is used for the analyzed sample, it shall be documented in the case file.

Y23 kit

1. Prepare a formamide/ILS mix for the appropriate number of wells as follows:
[(0.5 µL WEN ILS 500 Y23) x (# samples)] + [(9.5 µL formamide) x (# samples)]
Note: WEN ILS 500 Y23 is not the same ILS used in the PowerPlex Fusion 6C Product Preparation
2. Vortex for 10-15 seconds
3. Pipet 10 µL of formamide/ILS into each well
4. Add 1 µL of amplified sample (or Y23 Allelic Ladder Mix) to each well. Cover wells with septa.
5. Centrifuge the plate briefly to remove air bubbles.
6. Denature samples at 95°C for 3 minutes, then immediately snap cool for 3 minutes.

Note: It is recommended that only 3-4 injections of samples amplified with Y23 be run on the 3500 instrument in any given run, as some sample/ILS sloping may begin to occur the longer the CE plate is left on the instrument.

Optional: A stabilizer can be added to the formamide/ILS mix if run are anticipated to be over 3-4 injections. The preparation for the reaction mix with stabilizer is as follows:

1. Prepare a formamide/ILS/stabilizer mix for the appropriate number of wells as follows:

- [(0.5µL WEN ILS 500 Y23) x (# of samples)] + [(9.5µL of formamide) x (# of samples)] + [(0.5µL Y23 Stabilizer) x (# of samples)]
2. Vortex for 10-15 seconds to mix.
 3. Pipet 10.5µL of formamide/ILS/Stabilizer into each well.
 4. Add 1µL of amplified sample (or Y23 Allelic Ladder Mix) to each well. Cover wells with septa.
 5. Centrifuge the plate briefly to removed air bubbles.
 6. Denature the samples at 95°C for 3 minutes, then immediately snap cool for 3 minutes.

In addition to using injection times less than validated default injection times for amplicons with excessive peak heights (e.g. causing –A shoulder artifacts or saturation), a smaller amplicon volume as described above can be loaded, or a dilution in either formamide or TE up to 1:10 can be used. If any of these methods or combinations thereof is used for the analyzed sample, it shall be documented in the case file.

Y-STR Panels and Bins

Yfiler® panels and bins are included in the GeneMapper® ID-X software as “Yfiler_v1.2X”. Modify the Marker Specific Stutter Ratios to those established by the WSP validation study. (See Yfiler® interpretation guidelines for stutter.) Open the panel and check all the boxes under the “Y Marker” column except for locus DYS385.

Y23 kit

GeneMapper® ID-X requires chemistry-specific panels and bins. For Y23, these have been acquired from Promega and imported into GMID-X. In the Panel Manager, check the “Y Marker” boxes for all loci except for DYS385.

Y-STR GUIDELINES FOR EVALUATING GENEMAPPER® DATA

Follow the guidelines and work flow provided for the analysis of autosomal STR data with the addition of the following information for interpreting Y-STRs.

Evaluation of Quality Control Samples

Amplification controls and the allelic ladder are reviewed under the Allelic Ladder Quality and Control Quality sections of the Analysis Summary tab. Verify that the allelic ladders and controls have passed the quality system criteria. Passing data does not need to be manually reviewed; the non-passing data shall be manually reviewed. Analysis of negative controls utilizes the GMID-X software as an expert assistant and ensures that any evidence of contamination that may be present within a negative control will be flagged by the software for the analyst to review.

Evaluation of Y-STR Profiles

Designation of True Alleles

All non-artifact peaks that are sharp, distinct and greater than or equal to analytical threshold will be considered alleles, documented, and considered during profile determination. Peaks greater than or equal to the reporting threshold for Yfiler® or analytical threshold for Y23 are alleles that can be considered for inclusionary purposes and statistical assessment. (See Y-STR Mixtures for further interpretation requirements for the reporting of major and minor components.)

The determination of a profile or individual genotype is aided by the use of thresholds established via validation.

Yfiler®

- **Analytical Threshold: 100 RFU**
The analytical threshold is the level at which a peak can be reliably differentiated from background fluorescence as either an allele or artifact as determined via validation.
- **Reporting Threshold: 200 RFU**
If an allele meets or exceeds the reporting threshold, it can be considered for inclusionary purposes and included in the statistic. The reporting threshold aids in the determination of genotypes within a profile.

Y23

- **Analytical Threshold: 100 RFU**
The analytical threshold is the level at which a peak can be reliably differentiated from background fluorescence as either an allele or artifact as determined via validation.

The appropriate analysis method shall be applied to the analysis of samples.

- Evidence samples shall be analyzed with peak detection set at 100 RFU.
- Reference samples shall be analyzed with peak detection set at 200 RFU.

Determination of a Single Source Y-STR Profile

Yfiler®

A Yfiler® genotype result can be considered to be from a single contributor if there is no more than 1 allele that is greater than or equal to the analytical threshold at each locus. The exception is the duplicated locus DYS385 which will often exhibit the presence of two balanced alleles for a single individual. If the Yfiler® genotyping result is determined to be from a single contributor, alleles that meet or exceed the reporting threshold can be reported in the Yfiler® genotype profile and be used for inclusionary purposes. If a Yfiler® genotyping result is determined to be from a single contributor, alleles between the analytical and reporting threshold may be used cautiously for exclusionary purposes.

Considerations for utilizing data less than the reporting threshold for exclusions should include RFU levels, total number of loci with data, and the presence of drop-out, inhibition or degradation.

Y23

A Y23 DNA typing result can be considered to be from a single contributor if there is no more than 1 allele (single copy) that is greater than or equal to the analytical threshold at each locus. The exception is the duplicated locus DYS385 (multi-copy) which will often exhibit the presence of two balanced alleles (>50% for peaks >600RFU) for a single individual. If the Y23 DNA typing result is determined to be from a single contributor, alleles that meet or exceed the analytical threshold can be reported in the Y23 profile and be used for inclusionary and exclusionary purposes. If two alleles are observed above the analytical threshold at DYS385, they may be used for inclusionary and exclusionary purposes. If only the allele is observed at DYS385, it must be equal to, or greater than, 600 RFU to be used for inclusionary purposes. If a single allele is observed below 600 RFU, drop-out of a second allele is possible so the single visible allele should only be used for exclusionary purposes.

Note – DYS389I/II is also duplicated locus (multi-copy) but looks like a single copy result as it produces a single peak for DYS389I and a single peak for DYS389II with the Y23 kit.

Interpreting Mixed Y-STR Profiles

The Yfiler® or Y23 DNA typing result is considered to be from a mixed source if two or more alleles are present at two or more single copy loci. More than 2 alleles present at DYS385 would indicate a mixture. The analyst should also consider the presence of multiple stutter peaks that are greater in percentage than what has been established in the validation. It should also be taken into consideration that loci other than DYS385 can experience duplications that will produce more than 1 allele.

Mixtures with three or more contributors are generally un-interpretable; however, a major component can sometimes be present. If a major component is suspected, interpretation of the sample can be done. Consultation with and approval by the DNA Technical Leader, Supervisor, or Technical Lead following agreement between analyst and reviewer shall be documented in the case file. Evaluation of these profiles must account for potential allele stacking.

Considerations for the Occurrence of Duplications

Typically a single copy Y-STR locus will exhibit a single PCR product. There are instances of duplications of Y-STR loci where more than one peak will appear (or more than two peaks will occur for the multi-copy DYS385). The possibility of duplications may present potential complications for determining if a Y-STR profile is a single source contributor or a mixed sample. The following are some guidelines to aid in this evaluation:

- The greater the number of loci with multiple alleles, the more likely it is a mixture.
- If there is more than one locus with multiple alleles, the further apart these loci are physically located on the Y chromosome, the more likely it is a mixture.
- Duplications will typically have a single repeat difference. Greater than a single repeat difference may indicate a mixture.

The alleles at duplicated and multi-copy loci, such as DYS385, should be treated as genotypes and documented in the allele table as such. (Example: 11, 14)

Variant Alleles

If a variant allele or multi-allele (i.e. duplication) pattern occurs, the sample will be re-injected to confirm the result. Alternatively, other matching samples from the same contributor can also qualify as confirmation. The analyst will also check the NIST STRbase web site (http://www.cstl.nist.gov/biotech/strbase/var_tab.htm), to see if the variant has been previously reported. If the variant has been previously reported, a printout of this information will be placed in the case file. Variant alleles confirmed in the WSP system and not previously seen should be reported to the NIST STRbase web site using the appropriate online procedures. A Y-STR variant is calculated in the same way as an autosomal variant, keeping in mind that not all Y-STR loci are tetranucleotide repeats. Y-STR duplications and deletions are not considered variant alleles but should still be reported to STRbase if not previously reported.

A variant allele present in an uninterpretable profile or at RFU levels consistent with the uninterpretable portion of a mixture do not need to be confirmed.

Anticipated Artifacts

In addition to the normal artifacts (spikes, dye blobs, pull-up, and –A shoulders) that occur in autosomal STR's, the following should be taken into consideration:

Stutter

In addition to the normal one repeat smaller stutter usually seen, the occurrence of the following can occur:

- The presence of plus stutter is more prevalent than in autosomal STR's and occasional N+4 stutter can occur.
- For Y23 increased forward stutter (i.e., n+4 and n+8) can be observed at the DYS389I and DYS389II loci when using the cell-line derived 2800M Control DNA. This forward stutter is inherent to this cell line DNA and is not seen in amplification of DNA from human blood, body fluid, or other human-derived samples.

Yfiler® - The following stutter threshold percentages established during validation will be used:

LOCUS	N-4 stutter	Additional Locus Specific Stutter		
DYS456	15%			
DYS389I	9%			
DYS390	13%			
DYS389II	16%			
DYS458	14%			
DYS19	9%	(N-2) ¹ 9%	(N+2) ¹ 4%	(N+4) 5%
DYS385 a/b	14%			
DYS393	11%			
DYS391	11%			
DYS439	9%			
DYS635	12%			
DYS392	-	(N-3) ² 16%	(N+3) ² 7%	
Y GATA H4	10%			
DYS437	8%			
DYS438	-	(N-5) ³ 4%		
DYS448	-	(N-6) ⁴ 6%		

¹N-2 and N+2 stutter is set at 1.5 to 2.5 bp range. ²N-3 and N+3 stutter is set at 2.25 to 3.75 bp range.

³N-5 stutter is set at 4.35 to 5.75 bp range. ⁴N-6 stutter is set at 5.25 to 6.75 bp range.

Y23 - The following stutter threshold percentages established during validation will be used:

Locus	N-4	N-8	N+4
DYS576	16%	2%	4%
DYS389 I	8%		
DYS448	(N-6) 4%		
DYS389 II	16%	2%	
DYS19	(N-2) 10%	(N-4) 11%	(N+2) 4%
DYS391	10%		
DYS481	(N-3) 28%	(N-6) 6%	(N+3) 5%
DYS549	12%	1%	2%
DYS533	12%		3%
DYS438	(N-5) 5%		
DYS437	7%		
DYS570	15%	1%	3%
DYS635	12%		4%
DYS390	12%		
DYS439	11%		4%
DYS392	(N-3) 15%		(N+3) 10%
DYS643	(N-5) 5%		
DYS393	15%	2%	3%
DYS458	17%	2%	2%
DYS385 a/b	16%	3%	4%
DYS456	15%	1%	6%
Y-GATA-H4	11%		3%

N-2 and N+2 stutter is set at 1.25 to 2.75 bp range

N-3 and N+3 stutter is set at 2.25 to 3.75 bp range

N-4 and N+4 stutter is set at 3.25 to 4.75 bp range

N-5 stutter is set at 4.25 to 5.75 bp range

N-6 stutter is set at 5.25 to 6.75 bp range

N-8 stutter is set at 7.25 to 8.5 bp range

Effects from Female DNA

Non-specific amplification of female DNA in samples with high levels of female DNA may occasionally be seen. These amplification products will normally not affect the interpretation of the Y-STR profile. The known female reference associated with a case sample can be amplified to aid in the interpretation of non-specific amplification products if needed.

Validation studies show that there is a correlation between the amount of female DNA in a sample and the performance of a Y-STR amplification. An increase in the amount of female DNA in a sample will generally result in a decrease of peak heights in the Y-STR profile. Samples with extreme levels of female DNA could result in complete inhibition of the male DNA. This should be taken into consideration when making casework decisions regarding choice of sample and combining of samples. The case sample extract can be diluted to aid in decreasing the amount of inhibiting female DNA if necessary.

Documentation of Artifacts

Artifacts meeting or exceeding analytical threshold will be documented for all questioned samples, reagent blanks, and the negative amplification control. For all known samples and the positive male amplification control, artifacts meeting or exceeding the reporting threshold (Yfiler)/reference analysis method threshold (Y23) will be documented.

Y-STR MIXTURE DECONVOLUTION

Mixtures with Major Components

When a major component can be distinguished from the mixture (generally a two person mixture), inclusions and exclusions can be made between known reference samples and the major contributor profile. A population database search to generate statistics can then be performed on the major profile. A major contributor will be deduced locus by locus. At each locus, if the allele from the tallest peak is greater than or equal to 400 RFU for Yfiler® or 800 RFU for Y23 and the shorter allele peak height is less than 50% of the taller allele peak height, then the tallest peak may represent the major contributor. A locus may only be included in the statistics when these conditions are met.

Caution should be used when interpreting DYS385 where each contributor may have up to 2 alleles. At this locus, alleles may only be attributed to the major contributor under the following circumstances:

- If 4 alleles are present, the two larger alleles must both meet the above guidelines in relation to the two smaller alleles.
- If only 3 alleles are present, one larger than the other two, the larger allele must be at least 800 RFU for Yfiler® or 1600 RFU for Y23 and the combined height of the two minor alleles must be no more than 50% of the height of the larger allele.
- If only 3 alleles are present, two larger than the other one, both the larger alleles must be at least 400 RFU for Yfiler® or 800 RFU for Y23 and the height of the smaller allele must be no more than 50% of the height of each of the larger alleles.
- If only 2 alleles are present, it is often not possible to determine the major contributor's profile due to significant peak height imbalance being observed in low level, single-source samples. The greatest imbalance observed during validation was the height of one allele being 35% (Yfiler®) or 32% (Y23) of the height of the second allele from the same contributor. Therefore, if the height of the smaller allele is no more than 30% of the height of the larger allele, and the larger allele is greater than or equal to 400 RFU for Yfiler® or 800 RFU for Y23, the larger allele may be attributed to the major contributor. Additionally, if both peaks are greater than or equal to 400 RFU for Yfiler® or 800RFU for Y23 and are within 50% or greater balance of each other, then both peaks can be attributed to the major contributor.
- If only 1 allele is present and it is greater than or equal to 400 RFU for Yfiler® or 800 RFU for Y23, this allele can be attributed to the major contributor.

Mixtures with a Minor Component

There will be no inclusions made or statistics generated for the minor component of a Y-STR genotype originating from more than two individuals.

When a minor component profile can be distinguished from a two person mixture, inclusions and exclusions can be made between known reference samples and the minor contributor profile. A population database search to generate statistics can then be performed on the deduced minor component. A minor contributor will be deduced locus by locus. Alleles may only be attributed to the minor contributor under the following circumstances:

Yfiler®

- 1) A reportable major contributor has been declared at the locus
- 2) The minor allele at the locus is greater than or equal to the reporting threshold
 - At those loci where only one allele has been identified, it can be assumed that the minor contributor has donated the same allele as the major contributor if it can be shown that the average percent contribution of the minor contributor would result in an allele that is greater than or equal to the reporting threshold at that locus. The average percent contribution will be determined from all loci where the major and minor contributors have separate alleles.

- Caution should be taken when considering a minor allele that is in the stutter position of the major allele and near the stutter percentage.
- 3) A minor contributor can be deduced at DYS385 using the following guidelines:
 - a. The deduced minor alleles all meet the reporting threshold
 - b. All of the interpretation guidelines previously outlined for deduction of a major component from DYS385 are also met.

Y23

- 1) A reportable major contributor has been declared at the locus
- 2) The minor allele at the locus is greater than or equal to the analytical threshold
- 3) If only one allele is present at a locus, the following steps should be taken to attempt to deduce the minor contributor's genotype:
 - a. The average percent contribution of the deduced contributor should first be calculated. This can be done by finding the loci in which the deduced contributor does not share with the assumable profile (i.e. a unique allele) and dividing the allele peak height by the total RFU at the locus. The calculated values from each locus should then be averaged.
 - b. The average percent contribution value in step1 should be multiplied by 30% to account for the maximum peak height variation observed in validation between a mixture of contributors at equal proportions. This value is the lowest expected deduced allele height percentage – referred to as the deduced allele estimate (DAE) percentage.
 - c. The DAE percentage will be used at the loci where only one allele is present. The total RFU observed at the locus will be multiplied by the DAE percentage to obtain the DAE peak height value. If the value is larger than the analytical threshold, the analyst can be confident that the deduced contributor's allele has not dropped out.
 - d. The final step is to evaluate any stutter peaks present and determine whether or not the stutter peaks may possibly be masking a contributor.
 - i. If the peak in stutter position does not exceed the stutter filter and is less than the DAE, it can be assumed that the stutter is not elevated and that both contributors share the same allele.
 - ii. If the peak in stutter position does not exceed the stutter filter and is greater than the DAE, the minor contributor's allele may be masked; in this case the locus should be considered inconclusive for the minor contributor.
 - e. Extreme caution should be used when interpreting mixtures with a total male DNA input of 0.06ng or less. As the amount of DNA input decreases, stochastic and amplification artifacts increase. At these levels, highly elevated stutter peaks may be observed that appeared to be part of the deduced profile, but actually are not.
- 4) A minor contributor can be deduced at DYS385 using the following guidelines:
 - a. The deduced minor alleles all meet the analytical threshold. The calculated DAE, if used, should be halved.
 - b. All of the interpretation guidelines previously outlined for deduction of a major component from DYS385 are also met.

Mixtures with a Known Contributor

In instances where a contributor is known or expected to be present (such as intimate samples, consensual partners, etc.), subtracting out the known contributor's alleles can allow for determination of the remaining deduced probative profile. The remaining profile may be used for a population database search to generate statistics. At those loci where only one allele has been identified, it can be assumed that both the known and deduced contributors have donated the same allele if it can be shown that the average percent contribution of the deduced contributor (as determined from all loci where the known and deduced contributors have separate alleles), would result in an allele that is greater than or equal to the reporting (Yfiler®) or analytical (Y23) threshold at that locus. If the deduced contributor could be masked in stutter, the procedure for consideration of stutter outlined under "Mixtures with a Minor Component" should be followed.

Mixtures with Indistinguishable Components

No inclusions will be made to mixtures where a major, minor, or deduced component cannot be distinguished. This is due to the inability to provide an evidentiary statistical weight to indistinguishable Y-STR mixtures. These situations will be reported out as “inconclusive”. Exclusionary comparisons made to indistinguishable mixtures may be done with caution. Considerations for exclusionary comparisons should include number of contributors, RFU levels, total number of loci with data above reporting threshold, the presence of inhibition, degradation and the possibility of drop-out.

Y-STR GUIDELINES FOR REPORT WRITING

Y-STR reports will contain comparable information as outlined for autosomal STR DNA reports in the Guidelines for Report Writing. The specific guidelines for conclusions and report writing for Y-STR analysis are listed below. The Y-STR report must also contain a statement of the amplification kit used and a reference to the statistical database used (if applicable). STR and Y-STR results can be combined by multiplying the STR LR and the Y-STR match probability. The ancestry group with the most common STR LR from the major ancestry groups (Caucasian, African American and Southwest Hispanic ((STR)/(Y-STR))) will be chosen for combination then converted to and reported in Likelihood Ratio format.

- Statistics were calculated using the U.S. Y-STR Database, version used (optional), www.usystrdatabase.org.

Single Contributor Profile

Inclusion/Match

The Y-STR profile of the known reference matches the Y-STR profile of the questioned sample. There will be no forensically significant differences between the allele calls obtained from the reference sample and the questioned sample. If a full profile is not generated for the questioned sample, inclusions and a statistic can still be made to the reportable loci. This can be done as long as the statistics for the typing results providing the most genetic information are reported and a resulting statistic other than one can be provided.

- The Y-STR profile obtained from the (Item Description) matches the Y-STR profile of (Individual). Therefore, neither (Individual) nor any of his paternal male relatives can be excluded as the donor of the male DNA from this sample. This Y-STR profile has been observed (X) times in the U.S. Y-STR database and is not expected to occur more frequently than 1 in XXXX male individuals in the U.S. population.

LR

Hypothesis: A known contributor vs. an unknown contributor.

A Y-STR profile and autosomal STR profile from the same sample can be reported as a combined LR match statistic.

- The Y-STR and DNA profiles obtained from the (Item Description) match the Y-STR and DNA profiles of (Individual). Therefore, neither (Individual) nor any of his paternal male relatives can be excluded as the donor of the male DNA from this sample. It is XXX times more likely to observe this combined Y-STR and DNA profile if it originated from (Individual) (or a paternal male relative) than if it originated from an unrelated male individual randomly selected from the U.S. population.

Exclusion

If the Y-STR profile of the known reference does not match the Y-STR profile of the questioned sample at all loci (barring a scientific explanation), then the donor of the reference sample is excluded as the source of the questioned sample.

- (Individual) is excluded as the source of the male DNA obtained from (Item Description).
- The Y-STR profile obtained from (Item Description) is from an unknown male designated Individual A.

Mixed Y-STR Profiles

- A Y-STR profile and an autosomal STR profile from the same mixture sample might be eligible to be reported as a combined LR match statistic. Contact the DNA Technical Leader for assistance.

Mixtures with a Deduced Major Component

- The Y-STR profile obtained from (Item Description) is of mixed origin consistent with having originated from two (or at least (#)) male individuals. The profile of the major component matches the Y-STR profile of (Individual). Therefore, neither (Individual) nor any of his paternal male relatives can be excluded as the major contributor of male DNA from this sample. This major Y-STR profile has been observed (X) times in the U.S. Y-STR database and is not expected to occur more frequently than 1 in XXXX male individuals in the U.S. population.
- (Individual) can be excluded as being the major/substantial contributor of the Y-STR profile obtained from (Item Description).

Mixtures with a Deduced Minor Component

- The Y-STR profile obtained from (Item Description) is of mixed origin consistent with having originated from two (or at least (#)) male individuals. The profile of the minor component matches the Y-STR profile of (Individual). Therefore, neither (Individual) nor any of his paternal male relatives can be excluded as a minor contributor of male DNA from this sample. This minor Y-STR profile has been observed (X) times in the U.S. Y-STR database and is not expected to occur more frequently than 1 in XXXX male individuals in the U.S. population.
- (Individual) can be excluded as being the contributor to the minor component of the Y-STR profile obtained from (Item Description).

Mixtures with an Expected Y-STR Profile Present

- The Y-STR profile obtained from (Item Description) is of mixed origin consistent with having originated from two (or at least (#)) male individuals. Assuming a contribution from (Individual) (or Assuming that (Individual) is one of the sources of the male DNA in this profile), then the profile of the other contributor matches the Y-STR profile of (2nd Individual). Therefore, neither (2nd Individual) nor any of his paternal male relatives can be excluded as a donor of the male DNA from this sample. This Y-STR profile has been observed (X) times in the U.S. Y-STR database and is not expected to occur more frequently than 1 in XXXX male individuals in the U.S. population.
- The Y-STR profile obtained from (Item Description) is of mixed origin consistent with having originated from two (or at least (#)) male individuals. Assuming a contribution from (Individual) (or Assuming that (Individual) is one of the sources of the male DNA in this profile), then the profile of the other contributor is an unknown male designated Individual A. (Individual) is excluded as a source of the human male DNA obtained from (Item Description).

Inconclusive/No Conclusion/No Result (Single Source and Mixtures)

When incomplete results are obtained, inclusions and exclusions may still be possible but caution should be used. Peaks below the reporting threshold (Yfiler®) cannot be used for inclusions but can be carefully considered for exclusions. Sometimes, no conclusions can be made due to the complexity or the limited

genetic information of the Y-STR profile. Additionally, no inclusions can be made to a mixture where a major, minor, or deduced component cannot be determined. The above situations may require one of the following statements:

- The (partial or trace) Y-STR profile obtained from (Item Description) is of limited genetic information to which no comparisons can be made.
- No Y-STR profile was obtained from (Item Description).
- No meaningful inclusions can be made to the Y-STR profile obtained from (Item Description), however (Individual) is excluded as a source of this profile.
- Due to the complexity (or limited information) of the mixed Y-STR profile obtained from (Item Description), no meaningful comparisons can be made.

Y-STR STATISTICAL CALCULATIONS

Y-STR loci are located on the non-recombining region of the Y chromosome and are considered linked. Statistics for Y-STR analysis are calculated using the counting method to determine the number of times a particular haplotype has been observed in a given population database. The frequency of the haplotype in the database is then estimated by dividing the number of times a haplotype is observed by the total number of haplotypes searched. The current version of the United States Y-STR Database maintained by the National Center for Forensic Science in Orlando, Florida will be used for this statistic. The most current version of the U.S. Y-STR database can be found online at www.usystrdatabase.org. This US Y-STR population database contains profiles typed with different multiplexes, containing different numbers and/or sets of loci, such that only a subset of the database may have been typed at all of the loci present in the evidence profile. This can lead to a seemingly paradoxical situation: a more discriminating set of loci gives a less discriminating frequency estimate than a less discriminating set of loci, simply because there were fewer database profiles typed with the more discriminating set. Consequently, for profiles that match in the complete locus set, it is acceptable to perform a population database search using a reduced locus set in an attempt to obtain the maximal discrimination potential. For matching profiles obtained in PowerPlex® Y23, the Yfiler® locus set can be used for population database searches until Y23 profiles populate the database in comparable numbers.

Single source, major components, minor components, and deduced profiles will be searched in the population database using the directions outlined on the website.

A 95% upper bound confidence interval will be placed on the haplotype frequency using the Clopper and Pearson method as per the current SWGDAM Y-STR guidelines. The application of a confidence interval corrects for database size and sampling variation.

- If the haplotype has not been previously observed in the database, the formula used for calculating the upper 95% confidence limit is:

$$1 - (0.05)^{1/n} \quad n = \text{database size}$$

- If the haplotype has been observed in the database, the formula used for calculating the 95% confidence limit can be found on the US Y-STR database website.

The number of observed matches in the database (haplotype frequency) with the 95% upper bound confidence limit applied (profile probability) will be reported for the overall total number of haplotypes in the database. Statistics for the individual ethnic population groups will be maintained in the case file. The profile probability will be truncated using the same two significant figure method used for autosomal STR statistics.

In the situation where both an autosomal and a Y-STR profile have been obtained from the same sample, the product rule may be used to combine the autosomal STR LR and the Y-STR haplotype match probability. The ancestry group with the most common autosomal STR LR from the major ancestry groups (Caucasian, African American and Southwest Hispanic (STR)/ (Y-STR)) will be combined with its respective Y-STR haplotype match probability by multiplication (see Figure 1).

Results:

Ancestry	# of Haplotypes	# of Haplotypes with Selected Alleles	Haplotype Frequency ¹	Profile Probability ² [Frequency Upper Bound (95%)]	Theta	Match Probability ³
African American*	6234	0	0.000000	0.000480 1 in 2083	0.00020	0.000680 1471 times
Asian	3998	3	0.000750	0.001920 1 in 521	0.00020	0.002120 472 times
Caucasian	7449	0	0.000000	0.000402 1 in 2488	0.00020	0.000602 1661 times
Hispanic	4765	0	0.000000	0.000628 1 in 1592	0.00020	0.000828 1208 times
Native American	3561	0	0.000000	0.000840 1 in 1190	0.00060	0.001439 695 times
Total	26007	3	0.000115	0.000280 1 in 3571		

Figure 1: Y-STR Match Probability (red outline)

The Y-STR match probability is calculated on the upper bound 95% confidence interval (p_A , profile probability) in the manner specified in the SWGDAM Y-STR guidelines. This is done using the following formula and the evolutionary model where θ is a measure of normalized variance in allele frequency acting as a co-ancestry correction factor for subpopulation effects.

$$\Pr(A|A) = \theta + (1-\theta)p_A$$

A = haplotype of interest

$\Pr(A|A)$ = probability of observing the haplotype given it has already been observed

DNA EXTRACT AND WORK PRODUCT TRANSFER/RETURN

This procedure is used in conjunction with the VACUFUGE PROCEDURE – CONCENTRATION, PRESERVATION, AND RECOVERY OF DNA EXTRACTS/WORK PRODUCT procedure within this manual.

DNA extract(s) or work product(s) shall be preserved prior to transfer to another laboratory for additional analysis.

All DNA work product produced during sample analysis, including remaining DNA extracts from evidence (excluding reference samples), associated reagent blank(s), microscope slides, and sample cuttings or cellular material not subjected to DNA extraction, shall be preserved and returned to the submitting law enforcement agency as a new item.

The preserved/dried DNA extracts, cell pellets, and sample cuttings shall be sealed in a plastic or foil bag for transfer/return. The new item containing the preserved DNA extracts packet and other work product(s) will be created in LIMS and transferred/returned.

If there is only one case associated with a reagent blank(s), the reagent blank will be included with the DNA extract(s) to be transferred/returned. Each preserved DNA extract and associated reagent blank can be in a separate plastic or foil bag or they all may be placed in one bag. If there is more than one case associated with a reagent blank, the reagent blank will be preserved and retained at the lab at room temperature. Reagent blanks from extractions where there is no remaining DNA may be discarded.

The case notes and report will specify the new item(s) that contains the returned DNA extracts and/or other work product. Additionally, where possible, evidence packaging will be labeled (i.e. stickers or other method) to indicate that DNA work product is enclosed.

QUALITY ASSURANCE/QUALITY CONTROL

DNA ISOLATION AREAS

1. All solutions used in DNA isolation are prepared according to the protocols in the Reagent Preparation section of this manual.
2. All glassware to be used in preparation of critical reagents will be autoclaved. If a solution itself is to be autoclaved, the glassware used in its preparation need not be autoclaved. Pipette tips and microcentrifuge tubes that are sterilized (as in the manufacturing process) need not be autoclaved.
3. Gloves will be worn where appropriate.
4. Instruments used to cut or handle stains (scissors, forceps, scalpels, Harris punch) should be cleaned before each use. Cut each sample on a clean sheet of paper.
5. Use plugged pipette tips when pipetting any solutions.
6. Care should be exercised when inserting pipettors into reagent containers. Reagents should be poured from the reagent bottle into a smaller container from which pipetting should be done. Avoid touching the container with the barrel of the pipettor.
7. The work area should be cleaned after each use with 10% bleach followed by a rinse with water or wiped down with another appropriate cleaner.
8. The interiors of microcentrifuges should be cleaned periodically.
9. DNA from reference standards should be isolated at a different time or a different place than DNA from evidence samples.

PCR SET-UP AREA

1. Gloves will be worn when working in the PCR set-up hood.
2. Set up the PCRs according to protocol. Use only the pipettes dedicated to the PCR set-up area. Use sterile, plugged pipette tips. Dispose of pipette tips in waste container in hood.
3. Reference standards and evidence samples may be amplified together provided the following precaution is taken: Add DNA isolated from evidence samples to PCR tubes before reference standard DNA is brought to the set-up area. Make sure each reaction tube is sealed after the addition of DNA.
4. The hood will be cleaned on a regular basis with a 10% bleach solution (or other appropriate cleaner) and then rinsed with water or wiped down with another appropriate cleaner. Pipette barrels will also be wiped regularly with a 10% bleach solution (or other appropriate cleaner). Additionally, the UV light in the hood (if so equipped) can be left on a few hours after each use of the hood and pipettes.

AMPLIFICATION AND PRODUCT ANALYSIS AREA

1. All solutions used in the amplification and product analysis area are prepared according to the reagent manual protocol.
2. Gloves will be worn where appropriate when working in the amplification room.
3. Only dedicated lab coats will be worn when working in this area.
4. Handling of PCR products will be done with dedicated pipettors and plugged pipette tips.
5. The LABMAT™ benchtop protective paper will be changed on a regular basis when necessary.
6. Any PCR product or other items contaminated with PCR product will be discarded in the appropriate container in the PCR amplification/analysis work area.
7. PCR product may only be stored in a dedicated refrigerator or freezer.

CONTROLS AND STANDARDS

Reagent Blank

A reagent blank(s) will be prepared each time a set of DNA samples is extracted. There must be sufficient reagent blank(s) present to accommodate all amplification volumes and kits run with the extraction set; therefore, it may be appropriate to have multiple reagent blanks. The reagent blank will be prepared like the other samples being extracted, except no source of DNA will be added to this sample. For differential extractions, a reagent blank corresponding to each fraction shall be generated: one corresponding to the sperm fraction and the other corresponding to the non-sperm fraction. Any generated reagent blank shall be amplified using the same primers, instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA. If only one kit is amplified, then only reagent blanks for that kit need be run. If multiple kits are amplified, then the appropriate reagent blanks for each kit must be run. The amplified reagent blank shall be typed using the same instrument model, injection conditions and most sensitive volume conditions of the extraction set.

If multiple reagent blanks are used within an extraction set, each reagent blank must be quantified and at least one must be amplified if any of the specimens associated with the extraction set will be amplified. If multiple reagent blanks are used and quantified within an extraction set, at a minimum, the reagent blank that demonstrates the greatest signal (if any) must be amplified and characterized.

If a reagent blank associated with an extraction set or sample being amplified is depleted, an analyst shall not continue on to a different amplification test kit without a reagent blank. The volume of sample amplified cannot exceed that of its reagent blank. Follow the analysis completed retention policy for the samples associated with the depleted reagent blank.

For samples extracted prior to July 1, 2009, every attempt shall be made to follow the above guidelines regarding the amplification of a reagent blank with its corresponding extracted DNA sample. However this may not always be possible. A sample extracted prior to July 1, 2009 may be amplified with a different amplification test kit even if the associated reagent blank has been previously depleted. If any volume of reagent blank remains, it shall be amplified with the new kit. In those cases where the reagent blank has been partially or fully consumed by past analysis with similar technologies (i.e., PCR), the analyst should make every attempt to ensure that there were no past issues with the reagent blank (contamination or otherwise). This information shall be documented in the case file.

Positive and Negative Controls

At the amplification step, two extra tubes containing the amplification master mix will be prepared. To one tube, the appropriate volume of positive control DNA (9947A or 2800M) will be added, and to the other an equal volume of TE or amplification grade water will be added.

A positive and negative control will be included for each kit run in every set of amplifications conducted.

Internal Size Standards

An appropriate internal size standard will be included with every sample prepared to be run on the CE unit. The data collected from this size standard will be used to verify the quality of a particular injection and also be used to estimate the size of any DNA fragments present in the run. It is essential to have this data in order to process samples in the GeneMapper® ID-X program.

Allelic Ladder

An appropriate ladder will be included with every set of samples run on the CE unit.

Substrate Sample

Substrate samples can, on occasion, provide useful information in an analysis. However, the relevance of the data generated by a substrate sample may not be intuitively obvious. Any data generated from a substrate sample should be evaluated very carefully as to its significance. The fact that DNA consistent with another individual is recovered in a "substrate sample" is not a clear indicator of the involvement of that individual in the particular incident under investigation. A substrate sample may be included in the samples run at the discretion of the analyst. However, if one is run, the analyst should be very clear as to the purpose of this sample, how it bears on the analysis and investigation, and what the significance of the results are. This shall be clearly documented in the analyst's notes.

REAGENT QUALITY CONTROL

QUALITY CONTROL TESTING

All reagents used in DNA analysis are evaluated routinely. The reagent blanks and positive and negative controls used during testing continually verify the suitability of the reagents.

In addition, the following reagents need to be evaluated prior to use in casework:

- Proteinase K
- Phosphate buffered saline (PBS)
- Dithiothreitol (DTT)
- Phenol/Chloroform/Isoamyl alcohol
- 20% Sarkosyl
- TE
- Tris/EDTA/NaCl (TNE)
- Sperm wash buffer (SWB)
- QIAGEN Buffer MTL
- QIAGEN EZ1® DNA Investigator kit
- Promega SwabSolution™ Kit
- Promega Plexor® HY System
- Promega PowerQuant™ System
- Promega PowerPlex® Fusion 6C
- Promega PowerPlex® Y23
- AmpFtSTR® Yfiler® kit
- DNASTable®

See individual laboratory protocols for evaluation of all other reagents used in STR testing.

A known DNA sample should be used to perform the QC of a new lot of reagent, kit or kit component before it can be used for casework. The known DNA sample must be analyzed using the current laboratory protocols. The known DNA sample must type correctly and meet all data analysis parameters as required in the current laboratory protocols. An exception is the QC of quantification kits where a known DNA sample is not required to be typed. For QC of quantification kits the Standard Curve must meet all data analysis parameters as required in the current laboratory protocols. If the sample does not type correctly or the analysis parameters are not met, the reagent, kit or kit component must not be used for casework until the problem is resolved and/or new reagents or kit materials are obtained. Scientifically valid exceptions pertaining to meeting the analysis parameters can be made if approved by the DNA unit supervisor in consultation with the DNA Technical Leader. The successful completion of the QC shall be documented on the reagent, kit or kit component and on the appropriate Reagent QC form (maintained electronically).

Quality Control testing will be verified by a qualified DNA Forensic Scientist to ensure a quality DNA profile (or analysis parameters) was obtained, any negative controls are free of contamination, all electronic data is stored in the appropriate folder on the Data Repository, and the corresponding QC form is filled out completely and accurately. The verifier's initials will then be added to the QC form, which will then be saved as a read only .pdf file and stored with the QC data for that reagent.

REAGENT LOT NUMBERS AND EXPIRATION DATES

To facilitate traceability and quality control of reagents, the WSP DNA laboratories shall use the following conventions.

Critical reagents (internally prepared and commercially prepared) shall have an expiration date. If a commercially prepared reagent does not have a manufacturer-provided expiration date, an expiration date shall be assigned and will be three years from the received date. Critical reagents shall be checked prior to use in casework.

When a reagent (critical or not) has an expiration date, the expiration date shall serve as the lot number for the reagent (i.e., mmddyy).

For those reagents that do not have an expiration date, a lot number shall be assigned. The lot number can be a numbering system consisting of the last two digits of the year received followed by sequential numbering (i.e., yy-1, yy-2, yy-3, etc.) or using a lab-assigned expiration date to ensure turnover of the reagent.

The following table lists expiration dates for the reagents used in the DNA section. Reagents are stored at room temperature unless otherwise noted.

AmpliTaq Gold®	Mfr provided, -20°C
Dithiothreitol (1M DTT)	3 years (stored frozen)
Phenol Chloroform Isoamyl Alcohol	3 years, ~4°C
DNASTable®	Mfr provided, ~4° C
Phosphate Buffered Saline (PBS)	3 years
Plexor® Calibration kit, Set A	Mfr provided, ~20°C
Plexor® HY Male Genomic DNA Standard (provided with Plexor® HY System kit)	Mfr provided, -20°C upon receipt, ~4°C after initial use; do not refreeze
Plexor® HY System	Mfr provided, -20°C; short term storage (less than 1 week) at ~4°C; minimize number of freeze- thaw cycles
Promega PowerPlex® Y23	Mfr provided, -20°C upon receipt, ~4°C after initial use
Promega PowerPlex® Y23 Stabilizer	2 years or Mfr provided, 2°C - 10°C
Promega PowerPlex® Matrix Standard	Mfr provided, ~4°C
Promega PowerPlex® Fusion 6C amplification kit	Mfr provided, -20°C upon receipt, ~4°C after initial use
Promega SwabSolution™ Kit	Mfr provided, upon arrival thaw completely, ~4°C after initial use
Promega PowerQuant™ Calibration Kit	Mfr provided, -30°C to -10°C; short term storage (less than 1 week) at ~4°C; minimize number of freeze-thaw cycles
Promega PowerQuant™ System	Mfr provided, -30°C to -10°C; short term storage (less than 1 week) at ~4°C; minimize number of freeze-thaw cycles

Promega PowerQuant™ Male gDNA Standard (provided with PowerQuant™ System kit)	Mfr provided, -30°C to -10°C upon receipt, ~4°C after initial use; do not refreeze
Proteinase K (from EZ1 DNA Investigator Kit)	Mfr provided
EZ1® DNA Investigator kit w/ carrier RNA and G2 Buffer	Mfr provided, cRNA frozen after reconstitution
Buffer MTL	3 years
20 % Sarkosyl	1 year
Sperm Wash Buffer (SWB)	1 year
Sterile Deionized Water (sdH ₂ O)	3 years
Sterile Distilled Water	3 years
TE Buffer	2 years
Tris/EDTA/NaCl (TNE)	1 year
Yfiler® amplification kit	Mfr provided, ~4°C
DS-33 Spectral Standard	Mfr provided, ~4°C

REAGENT PREPARATION

Use reagent grade chemicals unless otherwise noted. Premade, reagent grade chemicals can be substituted for many of the recipes below. Prepare all solutions using deionized water (dH₂O). Wear gloves and follow safety recommendations provided by the manufacturer for handling chemicals. Comply with any and all laws, regulations, or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste. Store all reagents at room temperature unless otherwise noted. Reagents are prepared as follows:

Table: Preparation of Reagents

Solution	Volume	Method of Preparation	Comments
1 M Tris-HCl, pH 8.0	1 L	<ol style="list-style-type: none"> 98 g Tris-HCl 46 g Tris base 800 ml of dH₂O Check pH [should be 8.0 (+/- 0.2)]. Adjust volume to 1 liter with dH₂O. Autoclave. 	<i>Stock solution</i>
0.5 M EDTA Disodium ethylene diamine tetraacetic acid dihydrate (Na ₂ EDTA·2H ₂ O)	1 L	<ol style="list-style-type: none"> 186.1g Na₂EDTA·2H₂O 800ml of dH₂O Stir with magnetic stirrer pH to 8.0 (+/- 0.2) by adding ~20g of NaOH pellets * Check pH (add 5N or 10N NaOH if needed) Adjust volume to 1 liter with dH₂O Autoclave or filter through a sterile 0.2 µm filter. <p>* The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.</p>	<i>Stock solution</i> Warning: EDTA is an irritant. Wear lab coat, gloves, mask, and protective eyewear. Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, and protective eyewear. Use caution when handling. NaOH, when combined with water, results in an exothermic reaction. Dissolve the NaOH pellets in the water gradually. Allow the NaOH to dissolve completely and cool between each addition.

Table: Preparation of Reagents (continued)

20% (w/v) SDS Sodium dodecyl sulfate	1L	<ol style="list-style-type: none"> 200 g electrophoresis-grade (ultra pure) SDS 800 ml dH₂O Adjust volume to 1 liter with dH₂O <p>-OR-</p> <p>SDS can be bought pre-made from SERI.</p>	<p><i>Stock solution</i></p> <p>Warning: Wear protective mask when weighing SDS.</p> <p>Warming (e.g. in a 37° C water bath) may be required to dissolve solids completely.</p>
TNE (TRIS/EDTA/NaCl) 10 mM Tris-HCl (pH 8.0) 100 mM NaCl 1mM EDTA (pH 8.0)	100 ml	<ol style="list-style-type: none"> Add 1 ml of 1 M Tris-HCl pH 8.0 (*stock solution) to approximately 75 ml of dH₂O. To this solution add 0.584 g NaCl and 200 µl 0.5M EDTA (*stock solution). Stir until dissolved. Adjust the pH to 8.0 (if necessary) with 0.4 N NaOH. Adjust volume to 100 ml with dH₂O. Autoclave. 	<p>Extraction reagent Expiration = 1 year</p>
20% Sarkosyl	100 ml	<ol style="list-style-type: none"> Add 20 g N-lauroylsarcosine to dH₂O Stir until dissolved Adjust volume of 100 ml with dH₂O Filter through a sterile 0.45 µM filter. 	<p>Extraction reagent Expiration = 1 year</p>
1.0 M Dithiothreitol (DTT)	10 ml	<ol style="list-style-type: none"> Add 1.542 g of DTT to 10 ml dH₂O Aliquot into 1 ml portions Store frozen 	<p>Extraction reagent Expiration = 3 years</p>

Table: Preparation of Reagents (continued)

Sperm Wash Buffer (SWB) 10 mM Tris-HCl, pH 8.0 10 mM EDTA 50 mM NaCl 2% SDS	500 ml	<ol style="list-style-type: none"> 5 ml 1 M Tris-HCl, pH 8.0 (stock solution) 10 ml 0.5 EDTA (stock solution) 1.46 g NaCl 50 ml 20% SDS (stock solution) 435 ml of dH₂O Check pH - adjust to 8.0 with HCl if needed Autoclave 	<p>Extraction reagent</p> <p>Expiration = 1 year</p>
Reconstituted carrier RNA	310µL	<ol style="list-style-type: none"> Add 310µL of autoclaved dH₂O to carrier RNA Vortex to ensure carrier RNA is thoroughly dissolved Aliquot into small portions for use Store frozen 	<p>Extraction reagent</p> <p>Lyophilized carrier RNA is provided as a component of the QIAGEN EZ1DNA Investigator Kit</p>
TE Buffer 10 mM Tris-HCl, pH 8.0 0.1 mM EDTA	1 L	<ol style="list-style-type: none"> 10 ml of 1 M Tris-HCl, pH 8.0 (stock solution) 0.2 ml of 0.5 M EDTA (stock solution) 990 ml dH₂O Check pH - adjust to 8.0 with HCl if needed Autoclave 	<p>Extraction reagent</p> <p>Expiration = 2 years</p>
Deionized Formamide Alternatively, the formamide can be aliquoted directly from the bottle and frozen.		<ol style="list-style-type: none"> 10 g of AG 501-X8 resin 100 g of formamide Stir with magnetic stir bar gently for at least 30 minutes Check pH using pH paper. <ul style="list-style-type: none"> If the pH is not greater than 7.0, decant the formamide Add an additional 10 g of resin. Stir for another 30 minutes. When the pH is greater than 7.0, aliquot into 1.0 ml portions. Store frozen. Throw away any tubes that do not freeze at –20° C. 	<p>Warning: Formamide should neither be breathed nor come in contact with the skin. Wear gloves and work in a fume hood.</p> <p>DO NOT remove the beads by filtration. The filter paper will dissolve in the formamide and replace any impurities which had been previously removed. Either decant it into a clean container, or let the beads settle out and aliquot directly from the container with the beads. The beads are coarse and will not pass through the opening of a 1 ml pipette tip.</p>

CALIBRATION OF INSTRUMENTS

Checking the calibration of equipment and instruments demonstrates they are working in the proper fashion and will produce the expected results. For each refrigerator/freezer/oven/water bath, variance should be established as per the Equipment Calibration and Maintenance section of the DNA Analysis Quality Assurance manual. If the instrument is functioning outside of its expected range, use of the instrument in forensic testing should be stopped immediately and measures taken to return the instrument to function within its accepted range. The following equipment/instruments may affect testing in DNA, and written documentation of their calibrations and checks (below) shall be maintained.

GENERAL

1. pH meters will be calibrated according to the manufacturers' instructions before they are used. The calibration including buffers used and, if recommended by the manufacturer, the slope shall be recorded.
2. Balances will be checked at least yearly by an external provider using NIST Traceable weights.
3. The operating temperature of refrigerators, freezers, and any other storage unit or area used to store reagents or evidence that would degrade if not maintained at or below a given temperature will be monitored and recorded each working day using the TempGenius system. If manual recording is necessary, a thermometer will be employed, and the temperature recorded each working day. Semiannually, the TempGenius sensors (or thermometers) used to record these temperatures will be verified against a NIST traceable thermometer. The sensor (or thermometer) can remain in service if it is within $\pm 4^{\circ}\text{C}$ of the NIST traceable thermometer. Alternatively, a thermometer can be used that is NIST traceable and certified for a specific time period as long as it is replaced or re-certified before that period has expired.
4. The operating temperature of ovens, water baths, or other apparatus that is used to maintain a constant temperature of reagents or a reaction shall be monitored and recorded each working day using the TempGenius system. If the oven, water bath, or other apparatus is intermittently used for reactions, the operating temperature shall be monitored and recorded prior to its use. If manual recording is necessary, a thermometer will be employed, and the temperature recorded each working day or prior to use. Semiannually, the TempGenius sensors (or thermometers) used to record these temperatures will be verified against a NIST traceable thermometer. The sensors (or thermometers) can remain in service if they are within $\pm 4^{\circ}\text{C}$ of the NIST traceable thermometer unless otherwise stated in the specific application protocol. Alternatively, a thermometer can be used that is NIST traceable and certified for a specific time period as long as it is replaced or re-certified before that period has expired.
 - a. The operating temperatures of apparatus used solely for non-critical processes (e.g. denaturing/snap-cooling of amplification product) do not require daily recording of temperatures. The sensors (or thermometers) of these apparatus will periodically be verified against a NIST traceable thermometer as described above.
5. On a bi-annual basis (2x per year), the thermal cyclers will be calibrated using the appropriate calibrating probe and the directions provided by the manufacturer for the temperature verification test and temperature non-uniformity test. In addition, the wells and heated cover will be cleaned bi-annually according to manufacturer specifications. If a contamination event occurs related to the thermal cycler, it shall be cleaned according to manufacturer specifications. When a new thermal cycler is installed, a performance verification plan addressing reliability will be drafted and submitted to the DNA Technical Leader for approval. If a thermal cycler fails calibration using the Driftcon™ temperature verification system, re-calibrate using a single probe digital thermometer.

The results of the performance verification tests will be summarized and retained in the instrument log book.

6. Annually the NIST traceable thermometer and the probes used to check the calibration of thermal cyclers will be verified by an appropriately qualified external agency.
7. Pipettes will have their calibration checked at least once a year by a qualified external agency.

The following instruments shall be performance checked prior to implementation for casework purposes and following routine maintenance or repair by qualified technicians:

- Qiagen EZ1 BioRobot
- ABI Prism 7500 Real Time PCR instrument
- ABI GeneAmp® PCR System 9700 thermal cycler
- Qiagen BioRobot Universal
- Qiagen QIAgility BioRobot
- Genetic analyzers

Records of the above checks and calibrations will either be maintained within the DNA sections or have a reference to where they are retained within the laboratory.

TEMPGENIUS – SETUP AND MAINTENANCE

INSTALLATION AND SETUP

The TempGenius system wirelessly records the temperature for refrigerators, freezers, water baths, and ovens in the laboratory. TempGenius provides constant monitoring of these units and if the temperature of a unit falls outside of a given range, will alert the user and record the event.

GENERAL INSTALLATION AND OPERATION

Refer to the TempGenius “Installation Guide and Operating Manual”.

SETTING UP ALERT PARAMETERS

Alert parameters for each sensor must be set-up separately. In order for an event to trigger an alert, a temperature outside the designated range must be maintained for the designated time “span”. For each sensor, parameters for three separate sets of alert criteria can be designated. Access the alert parameters for a sensor via Dashboard → Set-up button → Sensor Set-up tab.

ALERT SCHEDULES

Alerts can be delivered via phone/voicemail, fax, text, or email. A single user can be notified multiple ways if desired, though the schedule for each delivery method must be set-up separately. Any assigned user(s) will receive alerts for all sensors during their alert schedule. To set up an alert schedule for a user, navigate to the Alert Set-up tab via Dashboard → Set-up button → Alert Set-Up tab.

MONITORING AND ALERT RESOLUTION

TempGenius monitors temperatures and automatically sends alerts to the assigned user(s) and the TempGenius Dashboard.

ALERT RESOLUTION

When an alert is generated, a resolution (or “Corrective Action” as termed by TempGenius) shall be recorded. Alert resolutions are handled via the Corrective Actions tab in the Dashboard. Enter the resolution description and initials of the user in the highlighted fields, and click “Save”.

MAINTENANCE

SEMIANNUALLY

A NIST probe reading for each monitored unit will be recorded semiannually to ensure the accuracy of the temperature readings recorded by TempGenius. If the TempGenius reading varies from the NIST reading outside of the allowable range, another reading will be taken at a different time. If the TempGenius reading persists outside of range, the manufacturer will be contacted.

ANNUALLY

The manufacturer defines the annual calibration as the adjustment (or replacement) of a sensor due to temperature variability against a NIST probe. The average offset for a sensor will be determined based on the total readings for the year. If the annual average offset of a sensor is in excess of 4°C, an adjustment

is mandatory and the manufacturer shall be contacted. A laboratory may choose to make an adjustment if the offset between a sensor and NIST probe is +/- 1°C.

If any adjustment is made to the sensor, a reading with a NIST probe will be taken the following work day to verify the adjustment. All adjustments will be documented on the TempGenius maintenance sheet.

EVERY TWO YEARS OR AS NEEDED

Replace the batteries (1.5V Lithium AA batteries) in the sensors by unscrewing the transmitter cover.

DAYLIGHT SAVINGS TIME CHANGES

When a daylight savings time change occurs, a “write setup” from the Pointware program to the data receiver must be performed to sync the computer and device times. Before performing the write setup, ensure that the historical data collections are current. This can be done by making sure the time of “last contact” listed in the Pointware program (under the Point Managers tab) is the same as the current time on the computer.

Once the most recently collected data has been downloaded, perform the write setup in the Pointware program via the Point Managers tab → right-click on appropriate Point Manager Name → Push → Write Setup. Click “Yes” when the dialog box pops up to complete the task.

QIAGEN BIOROBOT EZ1® WORKSTATION – MAINTENANCE

AFTER EACH RUN

1. Clean the EZ1 worktable and cartridge rack with dH₂O followed by ethanol.
2. Close the workstation door and follow the prompts to clean the piercing unit.
3. Open the workstation door and wipe the piercing unit using a tissue moistened with dH₂O followed by a tissue moistened with ethanol.
4. Clean the metal plate underneath the EZ1 worktable with dH₂O followed by ethanol.
5. Close the workstation door.

ONCE MONTHLY (OR MORE OFTEN AS NEEDED)

Grease the O-rings:

1. Open the workstation door.
2. Remove the worktable and cartridge rack and push back the tray holder.
3. Add a small amount of silicon grease to each pipettor head O-ring using any method that minimizes the amount of grease added.
4. Wipe off any excess grease.

NOTE: One method that can be used is to add a small amount of silicon grease to the inside top edge of an unused filter-tip. Place this tip onto the pipettor head, and rotate the tip over the O-rings.

ANNUALLY

On a yearly basis, the BioRobot instruments will be serviced by a QIAGEN technician.

PERFORMANCE CHECK

A performance check shall be conducted prior to use on casework for each new EZ1 BioRobot® unit. This performance verification shall address reliability and test for contamination and shall be documented.

Following yearly maintenance or repair, the instrument will be performance checked by extracting/purifying a known sample and a reagent blank followed by quantification of the samples. The extracted samples shall yield sufficient DNA to target a robust amplification and the blank shall be free of DNA (i.e., Undet.); however, amplification is not required. If amplified, the known sample shall genotype correctly and the blank shall be free of DNA. The results of all performance verifications of the Qiagen BioRobot® workstations shall be summarized and maintained.

AB7500 REAL TIME PCR SYSTEMS INSTRUMENT – SETUP

CREATING DETECTORS – PLEXOR HY

Before creating a plate document, detectors shall be created in the SDS software. Once the detectors are created, this step may be omitted in subsequent runs.

1. Launch the SDS software and open a new plate document. The New Document Wizard dialog box will appear. Select Finish. Two caution dialog boxes will appear sequentially – on the first, click OK; on the second, click Cancel.
2. Select Tools > Detector Manager. In the lower left, select File > New. The New Detector dialog box will appear.
3. In the lower left of the Detector Manager dialog box, select File > New to open the New Detector dialog box. Create the following detectors.
 - a. Autosomal detector for the Plexor® HY System:
 - i. Enter “Autosomal” in the Name field.
 - ii. Select “FL” as the Reporter Dye.
 - iii. Ensure “(none)” is selected as the Quencher Dye.
 - iv. Click the Color square to select a color.
 - b. Y detector for the Plexor® HY System:
 - i. Enter “Y” in the Name field.
 - ii. Select “CO560” as the reporter dye.
 - iii. Ensure “(none)” is selected as the Quencher Dye.
 - iv. Click the Color square to select a color
 - c. IPC detector for the Plexor® HY System:
 - i. Enter “Plexor IPC” in the Name field.
 - ii. Select “CR610” as the Reporter Dye.
 - iii. Ensure “(none)” is selected as the Quencher Dye.
 - iv. Click the Color square to select a color.

To create another detector, select Create Another to add the detector and to reset the New Detector dialog box. Select OK and Done when detector creation is complete.

CREATING A PLATE DOCUMENT TEMPLATE

Plexor® HY System

A plate document template greatly reduces the time required to set up a plate document. Typically, the quantification standards and detectors will already be in place in a previously agreed upon layout requiring only that the unknown samples and detectors be entered. Additionally, thermal cycler conditions and reaction volume settings may be attached to a template. Templates may also be created for situations that consistently require a plate document that differs from a day-to-day standard plate layout. In all templates created for casework analysis, ensure that the DNA quantification standards are run in duplicate.

1. Launch the SDS software.
2. In the SDS software, select “File”, then “New”.
3. In the New Document Wizard window, select the following: **Assay:** Absolute Quantification (Standard Curve), **Container:** 96-Well Clear, and **Template:** Blank Document. Select “Next”.

4. Highlight the line with the appropriate detector name and reporter dye for the assay (Autosomal, Y, and Plexor IPC), then select “Add”.
5. Select “IC5” for the “Passive Reference” from the drop-down box. Select “Next”.
6. In the lower window, select the box above the ‘A’ in the table to select all wells. In the top window, check the box for each detector. Leave “Task” as “Unknown” for all detectors.
7. Select “Finish”.
8. Set thermal cycling program (refer to the Plexor® HY System Technical Manual).
9. Save the plate document as a template.
 - a. From the SDS software, select “File” then “Save As”.
 - b. For “Save as type”, select SDS Templates (*.sdt).
 - c. Locate and select the Templates folder within the SDS software folder (C:\Program Files\7500 System\Templates). Enter a template file name. This might be a generic name easily recognizable for subsequent runs or it might be a name specific to the plate set up.
 - d. Select “Save”.

PowerQuant™ System

A plate template file (.edt file) can be used to store the dye information, target names and tasks; well locations and DNA concentrations for the DNA standards; run method, and analysis settings.

In all templates created for casework analysis, ensure that the DNA quantification standards are run in duplicate.

1. Turn on the computer associated with the 7500 instrument. Turn on the 7500 instrument then launch the AB 7500 Software.
2. On the *Home* screen, select the *Advanced Setup* icon (or select “Advanced Setup” from the *New Experiment* button).
3. In the *Experiment Properties* form, specify the following parameters
 - Enter an appropriate name for the template
 - Select the following: “7500 (96 Wells)” for instrument, “Quantitation – Standard Curve” for experiment, “TaqMan® Reagents” for reagents to use, and “Standard (~2 hours to complete a run)” for ramp speed.
4. Select “Plate Setup” from the *Setup* menu in the left panel. Select the *Define Targets and Samples* tab. Click three times on “Add New Target”, and enter the following information:

Target Name	Reporter	Quencher
Autosomal	PQ_FAM	NFQ-MGB
Y	PQ_CFG540	NFQ-MGB
Degradation	PQ_Q670	NFQ-MGB
IPC	PQ_TMR	NFQ-MGB

5. Select the *Assign Targets and Samples* tab and highlight the wells in use on the *View Plate Layout* tab by dragging the pointer over the plate wells. Assign all four targets to the selected wells by selecting the boxes under “Assign”.
6. Highlight the wells containing the DNA standards on the *View Plate Layout* tab and select “S” as the Task for the autosomal, Y and degradation targets. The Task for the IPC should be “U”.

- Enter the concentration for each DNA standard in the Quantity field without the unit of measure (e.g. enter 50 for 50ng/μl).

Notes:

- Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time so that values need to be entered only once.
 - The *Define and Setup Standards* tool will not work for assigning a standard curve to multiple targets in the same sample.
- Select “PQ_CXR” from the *Select the dye to use as the passive reference* drop-down menu
 - From the *Analysis* menu at the top of the screen, select “Analysis Settings”.
 - On the *CT Settings* tab in the *Select a Target* panel, select the Autosomal target. In the *CT Settings for Autosomal* section, uncheck the *Use Default Settings* box and uncheck the *Automatic Threshold* box. Enter 0.2 for the threshold. Leave the *Automatic Baseline* box checked. Repeat this for each target with the following threshold values:

Autosomal:	0.2
Degradation:	0.2
IPC:	0.3
Y:	0.2
 - Select “Run Method” under “Setup” in the left panel and modify the default run method as follows:
 - Enter “20” for “Reaction Volume Per Well”
 - Select the first Holding Stage. Right click using the mouse and choose “Delete Selected” to delete this stage.
 - Change the single remaining Holding Stage to 2 minutes at 98°C.
 - Enter “39” for “Number of Cycles” under “Cycling Stage”
 - Change Step 1 to 98°C for 15 seconds and Step 2 to 62°C for 35 seconds
 - Ensure that the *Data Collection On* icon is active for Step 2 of the Cycling Stage

Note: The *Data Collection On* icon is shown in the legend at the bottom of the screen.

- Save the template to an appropriate location using the “Save as Template” option from the file menu. The instrument setup and thermal cycling program can be saved as a template for future use.

AB 7500 REAL TIME PCR SYSTEM INSTRUMENT – MAINTENANCE

The AB 7500 Real Time PCR System is a PCR instrument used for the quantification assay. The instrument has the following maintenance schedule.

On at least a yearly basis or if the instrument is moved to a new laboratory location, the following checks shall be performed: 1) the Regions of Interest (ROI) calibration, and 2) the Dye Calibration. These yearly checks may be performed by ABI service personnel as part of the regular instrument maintenance schedule.

After approximately 2000 hours of use, the bulb shall be replaced. The following checks shall be performed each time the bulb is replaced: 1) calibrate the ROI, 2) run a background calibration, 3) perform an Optical calibration and 4) run the Dye calibration.

BACKGROUND RUN/CHECK SAMPLE BLOCK FOR WELL CONTAMINATION

1. Turn on the 7500 instrument and launch the SDS Software.
2. Create a new background plate document by selecting File > New. Select 'Background' for the Assay, '96-Well Clear' for the Container, and 'Blank Document' for the Template. Click Finish.
3. Save the background plate as a SDS Document (*.sds).
4. Place either a 96-well background tray (provided in the Spectral Dye Kit) or a 96-well tray with each well containing 50ul of dH₂O or PCR buffer into the instrument. Shake the plate and centrifuge as necessary to remove bubbles.
5. In the background plate document, select the Instrument tab and click start.
6. The background run may take 10 minutes. Do not set up any new plate documents, open existing files, or perform other processes on the system during the background run to avoid compromising the background data file.
7. When the run is complete, select the Results tab to view the raw background data.
8. Select all wells, and view the background raw data from all 96 wells. The plotted lines should form a tight band with fluorescent signals for filters A, B, C and D below 72,000 fluorescent standard units (FSU) and filter E below 90,000 (FSU). Any outlier wells (signifying potential well contamination) shall be identified and cleaned using a swab with distilled/deionized water and (if needed) with a 10% bleach solution, followed by ethanol. The background run must be rerun to confirm whether the contaminant has been successfully removed.
9. Click Analysis then Extract Background.

Document the performance, result, and any actions taken for the background run. A service call may be necessary if the background for a well cannot be cleaned up.

MONITORING THE LAMP

1. Turn on the 7500 instrument and launch the SDS Software.
2. Click File > New to open up a New Document window.
3. Click Instrument and select Lamp Status/Replacement.
4. Check that usage (hours) is less than 2000 hours.

BIANNUAL

OPTICAL CALIBRATION

1. Turn on the 7500 instrument and launch the SDS Software.
2. Create a new background plate document by selecting File > New. Select 'Calibration' for the Assay; '96-Well Clear' for the Container; and 'Blank Document' for the Template. Click OK.
3. Save the optical calibration plate as an SDS Document (*.sds).

NOTE: Let the instrument warm up for 10 minutes before starting test.

4. Remove a ROI calibration plate from a spectral calibration kit (stored in freezer). Allow the plate to come to room temperature. Maintain the plate in the packaging until ready to use. Vortex the plate and centrifuge to ensure that no bubbles are in any of the wells.
5. Load the plate in the holder, ensuring it is properly aligned.
6. In the calibration plate document, select the Instrument tab. Click Start.
7. When the run is complete, click OK.
8. Click Start Calibration in the Optical screen (or select Analysis → Extract).
9. When the message is displayed indicating the extraction is complete, Click OK.

ANNUAL

DYE CALIBRATION – PLEXOR® HY DYE CALIBRATION PLATE

The instrument must be calibrated for fluorescein, CAL Fluor® Orange 560, CAL Fluor® Red 610, and IC5. Use Plexor® Calibration Kit, Set A, which includes aliquots of the four calibrators at 100X concentrations, along with a calibration buffer for use as a diluent to create a spectral calibration plate for each dye.

Note: This should also be performed before initial use of the Plexor® HY System and following bulb replacement

Creating a Plexor® HY Pure Dye Calibration Plate

1. Thaw the four Concentrated Calibrators and Calibration Buffer.
2. Vortex the Concentrated Calibrators and Calibration Buffer to mix.
3. For each spectral calibrator, dilute 20µl of Concentrated Calibrator in 1.980ml of Calibration Buffer.
4. Vortex the diluted spectral calibrators for 3-5 seconds to mix.
5. For each diluted spectral calibrator, dispense 20µl to all 96 wells of a 96-well optical plate. Mark the side of the plate skirt to designate the fluorescein, CAL Fluor® Orange 560, Cal Fluor® Red 610 and IC5 spectral calibration plates.
6. Apply a plate seal to each plate. Protect plates from light.
7. Centrifuge plates briefly.
8. Follow the protocol in the instrument installation and maintenance guide to perform the calibration with the four plates. When required, define fluorescein as "FL", CAL Fluor® Orange as "CO560", CAL Fluor® Red as "CR610", and IC5 as "IC5". These designations will be used during instrument programming, data import into the Plexor® Analysis Software and final reporting.

Performing a Dye Calibration - Plexor® HY

Ensure a background calibration has been done prior to dye calibration.

1. Turn on the 7500 instrument and launch the SDS Software.
2. Click File > New. In the New Document dialog box, select 'Pure Spectra' for the Assay, '96-Well Clear' for the Container, and 'Blank Document' for the Template. Click Finish.
3. Select the dye to be calibrated from the dye list and click Calibrate

NOTE: Upon removing the dye calibration plate from storage, vortex and centrifuge to ensure there are no bubbles.

4. Place the dye plate to be calibrated into the 7500 tray and click Yes on the popup window to start calibration run.
5. After the run has completed, verify the grouping of the dye spectra.
 - a. Select all of the wells of the plate in the plate layout on the Spectra tab.
 - b. For each spectrum in the raw data, verify that the fluorescence plot is:
 - i. Within the detectable range of the 7500.
 - ii. Free of irregular spectral peaks.
 - iii. Present in the correct channel for the dye.
6. Document the successful performance of the calibration. If any test fails, consult the 'Troubleshoot the Dye Calibrator' section of the Maintenance Manual.

NOTE: After calibration, store the calibration plate, protected from light, at -20°C. Reuse the plate when reanalysis is necessary.

Dye Calibration – PowerQuant™ Dye Calibration Plate

Before using the PowerQuant™ System, the instrument must be calibrated for FAM, CAL Fluor® Gold 540 (CFG540), TMR, Quasar® 670 (Q670), and CXR dyes. Use the PowerQuant™ System Calibration kit which includes each of the dyes at a 100X concentration along with a calibration buffer for use as a diluent to create a spectral dye calibration plate for each dye.

Prior to performing the PowerQuant™ dye calibration, perform the ROI calibration, background calibration and optical calibration as per this manual.

Note: This should also be performed before initial use of the PowerQuant™ System and following bulb replacement

Creating a PowerQuant™ Calibration Plate

1. Thaw the five PowerQuant™ Calibration Standards (FAM, CFG540, TMR, Q670 and CXR) and the Calibration Buffer.
2. Vortex the concentrated Calibration Standards and Calibration Buffer for 10 seconds to mix. DO NOT centrifuge the Calibration Standards after mixing.
3. Dilute each PQ Calibration Standard 100-fold in PQ Calibration Buffer in a separate tube as detailed in the table below.

Reagent	FAM	CFG540	TMR	Q670	CXR
---------	-----	--------	-----	------	-----

Cal Std FAM	22µl	--	--	--	--
Cal Std CFG540	--	22µl	--	--	--
Cal Std TMR	--	--	22µl	--	--
Cal Std Q670	--	--	--	22µl	--
Cal Std CXR	--	--	--	--	22µl
Calibration Buffer	2178µl	2178µl	2178µl	2178µl	2178µl
Total Volume	2200µl	2200µl	2200µl	2200µl	2200µl

4. Vortex the diluted Calibration Standards for 10 seconds to mix. DO NOT centrifuge after mixing.
5. Set aside five 96-well optical plates, each marked with a respective calibration standard. For each diluted Calibration Standard, dispense 20µl to all 96 wells of a 96-well optical plate reserved for the appropriate dye-specific plate.
6. Seal each plate with the an optical adhesive film. Protect plates from light.
7. Centrifuge plates briefly.

Note: Prepared calibration plates can be stored protected from light at -20°C for up to 4months.

Performing a Dye Calibration

Ensure a background calibration has been done prior to dye calibration.

The protocol followed can be found in the PowerQuant™ Technical Manual and is the Calibration protocol for the AB 7500 Software, version 2.0.6.

1. Turn on the computer associated with the 7500 instrument. Turn on the 7500 instrument, and then launch the SDS Software.
2. From the *Instrument* menu at the top of the screen, select “Instrument Maintenance Manager”. Select the *Dye* section from the left panel. Select “Custom Dye Calibration”, and choose “Start Calibration.”
3. In the *Dye Calibration* window that appears, select *New Dye* button. In the *Dye Library* window that appears, select the *New* button.
4. Enter PQ_FAM as the new dye name, select the *Reporter* radio button under “Type” and then select “OK”. Repeat this process to define the remaining dyes: PQ_CFG540, PQ_TMR, PQ_Q670, and PQ_CXR.
5. In the *Custom Dye Calibration Setup* window, select the dye to be calibrated from the *Dye Name* drop-down list.

NOTE: If using a previously prepared and stored calibration plate, vortex and centrifuge to ensure there are no bubbles.

6. Place the dye plate to be calibrated into the instrument. Select “Next”, then “Start Run”.
7. Once the calibration run is complete, select “Next” in the bottom right corner of the window. The software will display the spectra and indicate whether the spectra are acceptable under “Status”.
8. Select “Finish”, and repeat steps 5-7 for each plate to calibrate the other dyes.

Note: FAM should give highest signal in Filter 1, CFG540 should give highest signal in Filter 2, TMR should give highest signal in Filter 3, Q670 should give highest signal in Filter 5, and CXR should give highest signal in Filter 4.

9. Document the successful performance of the calibration. If the custom dye calibration fails, prepare new standard calibration plate(s) and recalibrate.

NOTE: After calibration, store the calibration plate for up to 4 months at -20°C, protected from light. Reuse the plate when reanalysis is necessary.

PERFORMANCE CHECK

For each new AB 7500 Real Time PCR quantitation instrument, a performance check shall be conducted prior to use on casework. This performance verification shall address sensitivity and shall be documented and retained in the instrument log book.

Following routine yearly maintenance, a performance check will be performed, documented, and retained and consist of the following:

1. Regions of Interest (ROI) and Dye calibration tests (may be run by service technician as part of maintenance) and
2. A quantification plate containing previously quantified samples to address reproducibility.

If an instrument is moved to a new laboratory location, the ROI and Dye calibration tests shall be performed.

PLEXOR® HY SOFTWARE SETUP

1. Launch the Plexor® Analysis Software.
2. Under the File menu, deselect “Set Passive Reference On Import”. The SDS software exports data that are already normalized to the passive reference. A separate import of the passive reference dye channel (IC5) is not necessary.
3. In the File menu, select “Import new Run” (icon may also be used). Select Instrument type “Applied Biosystems 7500 SDS v1.4 & prior”.
4. Select “Add Target” three times. Enter the target names (Autosomal, Y, and IPC) and select the corresponding dye from the drop-down menus (FL for Autosomal, CO560 for Y, and CR610 for IPC).

Note: The name of the dye must be the same as that in the original SDS data file. Ensure that “Amplification” and “Melt” are checked for all three targets.

QIAGEN BIOROBOT® UNIVERSAL – MAINTENANCE

When a maintenance procedure is due, the “Run” icon in the Execute Environment will be yellow. Selecting the Maintenance Environment will allow the user to view the current maintenance procedures that are due and all maintenance procedures available.

There are six categories of maintenance procedures: Regular, Daily, Weekly, Monthly, Biannual and Preventative. Because the BioRobot® Universal is not necessarily used on a daily basis, maintenance protocols that are due on a more frequent basis than the robot is used will be run before each use rather than by time. The exception to this is the Preventative maintenance procedure that is conducted by a Qiagen Service Representative.

An electronic log of the maintenance performed is maintained on the computer associated with the robot.

REGULAR MAINTENANCE

These are procedures that are typically run between protocol runs and are typically part of the pre or post run instructions. Certain of these procedures listed in the BioRobot® Universal Manual are not part of the laboratory’s protocols, therefore are not required.

DAILY

Empty, rinse and re-fill the system liquid container (if necessary) and system liquid bottle (if using). Wipe all parts of the tip-disposal station with a soft, lint-free tissue moistened with detergent. Remove removable objects from the worktable and wipe the worktable and objects with a soft, lint-free tissue moistened with ethanol-based disinfectant. Wipe with a soft tissue moistened with water and dry.

WEEKLY

If the Universal BioRobot® has not been used for 1 week, perform the following procedures:

- Select Tools > Reinitialize Robot
- Select Tools > Flush System
- Select Tools > Reinitialize Robot

The procedures listed in the BioRobot® Universal System Manual are for applications of the Universal that the laboratory is not using at this time and will not be performed.

MONTHLY

Perform the daily maintenance procedures before performing the monthly procedures.

Check the high-speed dispensing system and liquid detectors:

- From the Execute Environment, select **Maintenance Protocol UNIV** and follow wizard instructions.
- After emptying the system liquid container, rinse with detergent followed by three rinses of tap water and three rinses of deionized water.
- Wipe the interior of the cap and exterior of the intake tube with a lint-free tissue moistened with detergent. Rinse three times with tap water followed by three rinses of deionized water.
- Fill the system liquid container with deionized water, reconnect it to the robot and select Tools > Flush System.

- After removing the removable objects from the worktable, remove the tip-disposal station and soak it along with the removable objects in detergent. Rinse three times with tap water followed by three rinses of deionized water. Wipe off excess liquid and dry with lint-free tissues.
- Clear the Event Log file:
 - From the Configuration Environment, select **Environment/Settings** and in the Event Log panel of the 21 CFR Part 11 Support tab click the **Release Event Log** button.

BIANNUAL

Biannual syringe calibration programs should be run according to manufacturer instructions.

Check the diluter system:

- Select Tools > Flush System to flush the diluter system.
- Run the Absorbance Plate Test. See the BioTek® Plate Reader Operator's Manual for instructions.
- From the Execute Environment, select **Syringe Calibration Check UNIV** from the protocol selection box and click **Run**. Follow the wizard instructions.
- If the diluter system needs to be calibrated, select **Syringe Calibration Adjust UNIV** from the protocol selection box and click **Run**. Follow the wizard instructions.
- The BioTek plate reader is used for this calibration step and is operated through the Gen5 Software on the Universal computer with the following steps:
 1. At Gen5's Welcome screen, select **Read a Plate**.
 2. Select **Plate type** - 96 well plate.
 3. Click **Read** to open the 'Read Step' dialog.
 4. Select a **Read Type** – end point, a **Detection Method** – absorbance, and a **Read Speed** – normal. Define the **Wavelength** at which the plate will read – 405nm.
 5. Click **OK** to return to the Procedure dialog.
 6. Click **OK** again to save and close the Procedure dialog. The 'Plate Reading' dialog will open.
 7. Click **Read** to begin the plate read. Click **OK** when the 'Load Plate' dialog appears. The plate will be read.
 8. Select **Plate – File export**. Under 'matrix' select **405** and then **Add**. Under 'table' select **405** and then **Add**. Click **OK**.
 9. Save as "NewPlate.txt" to path C:\Anthos\
 10. Open NewPlate.txt in C:\Anthos\ using Word Pad. Place cursor to the left of the letter 'A' and press 'Enter' creating an empty row above. Save this change to the same file name.
 11. Click **Continue** in the QiaSoft™ software.

ANNUAL

PM as scheduled by a Qiagen Service Representative.

PERFORMANCE CHECK

A performance check of each new BioRobot Universal shall be conducted prior to use on casework. This

performance verification shall address reliability and test for contamination and shall be documented.

If a Qiagen technician performs any routine maintenance or repair, or a significant revision to the Universal BioRobot's programming is made, a performance check will be performed and documented. After a revision to the programming, the "Data" folder on the Universal BioRobot computer will be archived to removable media (i.e. CD, DVD, or equivalent technology). Two copies will be made. One copy will be retained in the laboratory, and the additional copy will be transferred to an offsite WSP Laboratory or separate secure location.

BIOTEK® PLATE READER – INSTRUMENT MAINTENANCE

BEFORE USE

1. Turn off and disconnect the instrument from the power supply.
2. Moisten a clean, lint-free tissue with water or with a mild detergent – do not soak tissue.
3. Wipe the plate carrier and all exposed surfaces of the instrument.
4. If detergent was used, wipe all surfaces with a tissue moistened with water.
5. Use a clean, lint-free tissue to dry all wet surfaces.

SEMI-ANNUALLY

1. Run the Absorbance Plate Test. Follow the procedure for the Absorbance Plate Test as outlined in the BioTek Plate reader user manual.
2. Run Liquid Test 1. Follow the procedure for Liquid Test 1 as outlined in the BioTek Plate reader user manual.

AS NEEDED

Replace the bulb. Follow the procedure for bulb replacement as outlined in the BioTek® Plate reader user manual.

ANNUALLY

The Absorbance Test Plate shall be sent out for re-certification.

QIAGEN QIAGILITY – MAINTENANCE

DAILY (FOLLOWING USE)

1. Remove all samples, plastic-ware and consumables from the instrument deck.
2. Empty discarded tips from the tip disposal box if needed.
3. Wipe the surfaces of the instrument deck, sample racks and plate trays with a towel moistened with a detergent solution. Alternately, the racks and trays can be rinsed with water and dried.
4. A UV decontamination step may be selected following the cleaning procedure.

YEARLY

On a yearly basis, the QIAgility BioRobot instruments will be serviced by a QIAGEN technician.

PERFORMANCE CHECK

A performance check of each new QIAgility BioRobot shall be conducted prior to use on casework. This performance verification shall address reliability and test for contamination and shall be documented.

Following yearly maintenance or repair, by a QIAGEN technician or a significant protocol revision a performance check will be performed and documented. The performance check shall consist of a set of known samples run through the appropriate protocols (quant set up or the CE plate set up for the instruments in the post PCR rooms) with the expected results obtained. Following a protocol revision, the former protocol will be removed and archived following the laboratory's data archival procedure (e.g. quarterly backup).

If the QIAgility pipettor head is replaced during the yearly maintenance or for a repair, the p-axis values may need to be verified against the Volume Calibration certificate for the new pipettor head. This can be done by selecting Option 1 of the Volume Calibration Wizard under Options/Robot Setup/Calibrate Volumes and checking that the P-axis values listed match those on the certificate. It may be necessary re-calibrate the plate positions and heights.

CALIBRATIONS

Calibrations are required following yearly maintenance or if changing the plastic-ware used. The software will indicate calibration is needed by displaying a red exclamation point in a box in the top right corner of the plate windows.

Calibrating plate position

Note: Tip Rack Holder calibrations must be performed before any other plate type can be calibrated.

Caution: The Y-arm will move during position calibration when the hood is raised. Do not click on any software buttons while parts of your body are within the instrument worktable.

1. Open the instrument hood and place the plates to be calibrated on the instrument worktable.
2. Ensure that tips are available in at least one Tip Rack Holder.
3. Open the plate position calibration screen by selecting "Options/Robot Setup/Calibrate plate positions".
4. Select a plate from the "Plate Selection" list. **Note:** Tip Rack Holders are listed as 96-well plates.
5. Use the "Lower Tip" and "Raise Tip" buttons in the "Tip Control" panel to move the pipetting head so that it is just above the plate surface.
6. Adjust the "X-position" and "Y-position" in the "Plate Origin" panel so that the pipetting head is directly above the center of the well indicated in red.

7. Click “Save.”

Calibrating Plate Heights

1. Ensure that tips are available, that the Tip Rack Holder is calibrated, and that the correct tips are set as available.
2. Place empty tubes in the first 4 wells of each plate.
3. Select “Options/Robot Setup/Calibrate plate height”. The “Configure Plate Heights” dialog box will appear.
4. Check the boxes next to the plates to be calibrated in the “Plates to Calibrate” list.
5. Define the number of wells from which to take an average. It is recommended that 4 wells are used to calculate the average.
6. Click on the “Autodetect (checked items)” button to initiate calibration and “Yes” to the “Ready to auto-detect well heights” in the pop-up window.
7. Click “Ok” to the auto-detecting heights for the 96 well plate...pop-up window.
8. If “Require confirmation from user?” was selected, the following message Bottom of well detected at xxx, based off the average of 4 samples” will appear. Click on the “Yes” button to accept the calibration, if the averages for a plate are within 10 units of each other. If the averages are not within 10 units of each other, click on the “No” button to reject the current calibration and then repeat steps 4-8.

AB 3500 GENETIC ANALYZER – SETUP AND MAINTENANCE

SETUP

SPATIAL CALIBRATION

The spatial calibration maps the positional relationship between the capillaries and the CCD camera. This calibration allows the instrument software to determine what fluorescent emissions are coming from which capillary. A spatial calibration must be done every time a capillary array is removed and replaced, the detector door is opened, the detection cell is moved, or the instrument is moved.

1. In the Maintenance navigation pane, select the spatial calibration.
2. Select **No Fill** or **Fill** to fill the array with polymer.
3. Start calibration.
4. Evaluate the spatial calibration. Ensure that you see
 - a. One sharp peak for each capillary (small shoulders are acceptable).
 - b. One marker (+) at the apex of every peak. No off-apex markers.
 - c. An even peak profile (all peaks about the same height).
5. Accept the results if the above criteria are met. Refer to the 3500 User Guide if the spatial calibration fails.

SPECTRAL CALIBRATION

The spectral calibration is applied during sample detection to calculate the spectral overlap and separate the raw fluorescent signals into individual color signals. Perform a spectral calibration after major maintenance on the system, such as changing the laser, calibrating or replacing the CCD camera, changing the polymer or capillary array, or if the instrument is moved to a new location. It may be necessary to repeat the spectral calibration if pull-up/pull-down increases or approximately every 6 months.

Fusion 6C

At first use, thaw the 6C Matrix Mix and Matrix Dilution Buffer completely. After first use, store the reagents between 2-10°C.

1. Pre-heat the oven to 60°C (at least 30 minutes prior to the first injection).
2. Vortex the 6C Matrix Mix for 10-15 seconds prior to use. Add 10µl of the 6C Matrix Mix to one tube of Matrix Dilution Buffer. Vortex for 10-15 seconds. The diluted 6C Matrix Mix can be stored for up to 1 week between 2-10°C.
3. Add 10µl of the diluted 6C Matrix Mix prepared in step 2 to 500µl of Hi-Di™ formamide. Vortex for 10-15 seconds.
4. Add 15µl of the 6C Matrix Mix with formamide prepared in step 3 to wells A1 through H1 of the 96-well plate. Briefly centrifuge the plate to remove bubbles. **DO NOT HEAT DENATURE.**

- Place the assembled plate on the instrument. Select the Spectral Calibration in the Maintenance navigation pane. Select “Matrix” for the chemistry standard and “Promega J6” for the Dye set. Ensure that the box is checked to allow borrowing.
- Start the calibration.
- Evaluate the spectral calibration data and accept or reject. Passing and failing capillaries are shown in green and red respectively. Borrowed capillaries are shown in yellow with an arrow indicating the capillary borrowed from.

NOTE: Refer to the PowerPlex® 6C Matrix Standard Technical Manual for instructions regarding creating a new Dye Set.

YFiler / Identifier Plus (HID Install Standard)

- Prepare the DS-33 Spectral Standard (final volume 100 µL)
 - 5 µL of Spectral Standard DS-33
 - 95 µL of Hi-Di Formamide
- Load 10 µL of standard solution into wells A1 – H1. Column 1 must be used – the software uses predetermined positions for the calibration.
- Heat denature the samples by placing in a thermal cycler or heat block set at 95°C for 3 minutes. Snap cool samples in a freezer for 3 minutes.
- Place the assembled plate on the instrument. Select the Spectral Calibration in the Maintenance navigation pane and ensure the appropriate calibration settings.
- Start the calibration. The software sets up 3 injections.
- Evaluate the spectral calibration data (see 3500 User Guide) and accept or reject. Passing and failing capillaries are shown in green and red respectively. Borrowed capillaries are shown in yellow with an arrow indicating the capillary borrowed from.

NOTE: If too strong, modify step 1 by adding 5 µL of Spectral Standard DS-33 to 195 µL of Hi-Di Formamide (final volume 200 µL).

Y23

At first use, thaw the 5C Matrix Mix and Matrix Dilution Buffer completely. After first use, store the reagents between 2-10°C.

- Pre-heat the oven to 60°C (at least 30 minutes prior to the first injection).
- Vortex the 5C Matrix Mix for 10-15 seconds prior to use. Add 10µl of the 5C Matrix Mix to one tube of Matrix Dilution Buffer. Vortex for 10-15 seconds. The diluted 5C Matrix Mix can be stored for up to 1 week between 2-10°C.
- Add 10µl of the diluted 5C Matrix Mix prepared in step 2 to 500µl of Hi-Di™ formamide. Vortex for 10-15 seconds.
- Add 15µl of the 5C Matrix Mix with formamide prepared in step 3 to wells A1 through H1 of the 96-well plate. Briefly centrifuge the plate to remove bubbles. DO NOT HEAT DENATURE.
- Place the assembled plate on the instrument. Select the Spectral Calibration in the Maintenance navigation pane. Select “Matrix” for the chemistry standard and “Promega G5” for the Dye set. Ensure that the box is checked to allow borrowing.

6. Start the calibration.

7. Evaluate the spectral calibration data and accept or reject. Passing and failing capillaries are shown in green and red respectively. Borrowed capillaries are shown in yellow with an arrow indicating the capillary borrowed from.

NOTE: Refer to the PowerPlex® 5C Matrix Standard Technical Manual for instructions regarding creating a new Dye Set.

MAINTENANCE

The Data Collection software installed on the computers associated with the genetic analyzers employs wizards to assist the user in maintenance procedures. Access the appropriate wizard and follow the prompts to complete the required general maintenance tasks.

Before each run

Check for bubbles in the pump block and all lines; remove using software wizards if necessary. Replace polymer, anode/cathode buffers if necessary.

Monthly

- Perform a water wash to flush the pump chamber and channels. This shall be done via the software wizard. The wizard requires Conditioning Reagent.
- Replace the polymer.
- Flush the water trap (pump trap).
- Replace the cathode buffer septa.
- Restart the instrument and computer.

Quarterly

- Run the HID Install Standard performance check (see 3500 User Guide). This verifies that the instrument conforms to fragment analysis sizing precision, sizing range, and peak height specifications. **NOTE:** The HID Install Standard requires a spectral calibration (as needed) using the AB DS-33 Spectral Standard – see 3500 User Guide.
- Backup run data and monitor the instrument database.

Annually

Preventative maintenance will be performed by a qualified technician. If an instrument is permanently taken out of service it does not require the annual maintenance to be performed in the year it is removed.

As needed:

- If polymer has been stagnant in the chamber/channels/array for an extended period between water washes and increased artifacts are observed, the array can be filled with fresh polymer (via the Fill Array Wizard) or a water wash can be performed.
- Replace the capillary array whenever poor precision or allele calling is noted, or when resolution or signal intensity decreases. It is acceptable to use a capillary array past the manufacturer's expiration date.
- Perform a spatial calibration whenever the capillary array has been removed/replaced, the detection cell door has been opened, the detection cell of the cap array is moved, or the instrument was moved.

- Perform a spectral calibration whenever a new capillary array is installed, a new dye set is used, after any optical alignment, or if pull-up/pull-down increases.
- Restart the instrument and computer.

PERFORMANCE CHECK

A performance check shall be done prior to use for casework purposes for each CE unit that is moved or when an AB technician provides repair or routine maintenance.

The performance check will consist of

1. HID Install Standard performance check (see 3500 User Guide) and
2. Successfully running a +C amplification positive or other known sample, an amplification negative, and a ladder on the instrument.

A new CE instrument also requires a 1 base pair precision check (± 0.5) with a minimum of data from 6 ladders run per capillary.

Verify that the sample genotyped correctly and meets all data analysis parameters as required in the current laboratory protocols. Documentation of the performance check will be recorded in the instrument log book.

A spatial calibration is required if repair/maintenance performed involved removing or replacing the instrument capillary (also if relocating the instrument). A spectral calibration is required if repair/maintenance involved the optics of the instrument (laser or CCD).

GENEMAPPER® ID-X SETUP

SIZE STANDARD

The size standard for use with PowerPlex® Fusion 6C and PowerPlex® is WEN_ILS_500_CS.

FUSION 6C PANELS AND BINS

GMID-X requires chemistry-specific panels and bins. For Fusion 6C and Y23, these have been acquired from Promega and imported into GMID-X. Custom panels and bins specific for STRmix analysis have been created from those acquired panels and bins from Promega. For Fusion 6C, in the Panel Manager, check the Y Marker boxes for DYS391, DYS576, and DYS570 to indicate they are Y markers. For Y23, check the Y Marker boxes for all markers except DYS385.

ANALYSIS METHODS

The Analysis Method defines peak detection, sizing, genotyping, and quality assessment parameters applied during the analysis of sample data. Instructions for creating Analysis Methods are below.

TO CREATE OR CHANGE AN ANALYSIS METHOD – FUSION 6C

1. In GeneMapper® ID-X, select GeneMapper® ID-X Manager.
2. Select the **Analysis Methods** tab, then click **New**.
3. Select the **General** tab.
 - a. Enter a name for the method. Instructions provided below for methods named
 - i. **Fusion6C_70rfu_1.2** (for unknown/question samples and negative controls)
 - ii. **Fusion6C_600rfu_1.2.1** (for known/reference samples, positive controls, and ladders).
 - iii. **Fusion6C_70rfu_STRMix** (for ladders and unknown/question samples for STRMix)
 - iv. **Fusion6C_600rfu_STRMix** (for ladders for STRmix, optional)
 - v. **Fusion6C_120rfu_DA** (for direct amplification reference samples (optional) and negative controls)
 - vi. **Fusion6C_500rfu_DA** (for direct amplification reference samples (optional), positive controls, and ladders (alternatively, the associated direct amplification ladder may be analyzed using Fusion6C_600rfu_1.2))
4. From the drop-down list for Security Group, select **GeneMapper ID-X Security Group**.

Note: Analysis of negative controls using the Fusion6C_70rfu or Fusion6C_120rfu_DA method utilizes the GMID-X software as an expert assistant and ensures that any evidence of contamination that may be present within a negative control will be flagged by the software for the analyst to review.

5. Select the **Allele** tab.
 - a. For **Fusion6C_70rfu_1.2**, **Fusion6C_600rfu_1.2.1**, **Fusion6C_120rfu_DA**, and **Fusion6C_500rfu_DA** methods
 - i. Select “PowerPlex_Fusion_6C_Bins_IDX-v1.2.1” from the Bin set drop-down menu.

- ii. Click on the box to select **Use marker specific stutter ratio if available**.
 - iii. All other boxes, including the Amelogenin Cutoff box should have “0.0” entered.
 - b. For **Fusion6C_70rfu_STRMix** and **Fusion6C_600rfu_STRMix** methods
 - i. Select “Fusion 6C Bins-STRMix” from the bin set drop-down menu.
 - ii. Click on the box to select **Use marker specific stutter ratio if available**. A custom stutter file has been created to remove non-standard stutters (n+/-2) without removing the standard stutter (n+/-4)
 - iii. All other boxes, including the Amelogenin Cutoff box should have “0.0” entered.
6. Select the **Peak Detector** tab.
- a. In the **Ranges** box, select **Full Range** from the drop-down menu. Select **Partial Sizes** from the drop-down menu; designate the start point at 60, stop point at 500.
 - b. In the **Smoothing and Baselining** box, select **Light** for Smoothing and type in “51” for the Baseline Window.
 - c. Select **Local Southern Method** in the **Size Calling Method**.
 - d. In the **Peak Detection** box, set the **Min. Peak Half Width** at 2 pts, the **Polynomial Degree** at 3, and the **Peak Window Size** at 15 pts; Set the **Slope Threshold Peak Start** and **End** both at 0.0.
 - i. For **Fusion6C_70rfu_1.2** and **Fusion6C_70rfu_STRMix** methods, set the peak amplitude thresholds at “70” for B, G, Y, R, and P dye filters and “600” for the O dye filter.
 - ii. For **Fusion6C_600rfu_1.2.1** and **Fusion6C_600rfu_STRMix** methods: set the peak amplitude thresholds at “600” for all dye filters.
 - iii. For **Fusion6C_120rfu_DA** method, set the peak amplitude thresholds at “120” for all dye filters.
 - iv. For **Fusion6C_500rfu_DA** method, set the peak amplitude thresholds at “500” for B, G, Y, R, and P dye filters and “120” for the O dye filter.
- NOTE: If a one base difference between two alleles cannot be resolved (i.e. minor peak 1 base from major peak) after two injection attempts, then the Polynomial Degree and Peak Window Size can be adjusted to help resolve that specific sample. This change of analysis parameters shall be documented in the case file and the modified analysis method shall be exported and maintained in the electronic case record.
7. Select the **Peak Quality** tab. In the **Min/Max Peak Height (LPH/MPH)** box, enter “600.0” for **Fusion6C_70rfu** method and enter “500.0” for **Fusion6C_120rfu_DA** and **Fusion6C_500rfu_DA** methods for both **Homozygous min peak height** and **Heterozygous min peak height**. Enter “1500.0” for **Homozygous min peak height** and “600.0” for **Heterozygous min peak height** for **Fusion6C_600rfu_1.2** method. Enter “40000” for **Max Peak Height (MPH)**. Enter “0.5” for Min peak height ratio in the **Peak Height Ratio (PHR)** box for **Fusion6C_70rfu** and **Fusion6C_600rfu** methods and enter “0.6” for **Fusion6C_120rfu_DA** and **Fusion6C_500rfu_DA** methods, “1.5” for Max peak width (basepairs) in the **Broad Peak (BD)** box, “2” for Max expected alleles in the **Allele Number (AN)** box, and “0.2” for the Cut-off Value in the **Allelic Ladder Spike** box.
 8. Do not alter the default settings in the **SQ and GQ Settings** tab.
 9. After completing these settings, click Save to save the changes and close the Analysis Method Editor.

The analysis method(s) can be exported to other copies of GeneMapper® *ID-X*.

GeneMapper® ID-X Parameters for the YFiler® Analysis Method

TO CREATE OR CHANGE AN ANALYSIS METHOD – Yfiler®

1. **General** tab.
 - a. Enter a name for the method. Instructions provided below for methods named or similarly named: “YFiler_100rfu_3500” (used for unknown/questioned negative control/reagent blank) and “YFiler_200rfu_3500” (used for known/reference, positive male control, and ladder samples).
 - b. From the drop-down list for Security Group, select **GeneMapper ID-X Security Group**.
 - c. Instrument type and a description may be entered.
2. **Allele** tab.
 - a. Select “AmpF!STR_Bins_v3X” from the Bin set drop-down menu.
 - b. **Use marker specific stutter ratio if available.**
 - c. Tetra: Global Minus Stutter Distance: From 3.25 To 4.75
Global Plus Stutter Ratio: 0.05
Global Plus Stutter Distance: From 3.25 To 4.75
 - d. All other boxes, including Amelogenin Cutoff box should have “0.0” entered.
3. **Peak Detector** tab.
 - a. In the **Ranges** box, select **Partial Analysis Range** from the drop-down menu; designate the start point at 2800, stop point at 10000 (this can be adjusted depending on run conditions). Select **Partial Sizes** from the drop-down menu; designate the start point at 80, stop point at 400.
 - b. In the **Smoothing and Baseline** box, select **Light** for Smoothing and type in “51” for the Baseline Window.
 - c. Select **Local Southern Method** in the **Size Calling Method**.
 - d. In the **Peak Detection** box, set the **Min. Peak Half Width** at 2 pts, the **Polynomial Degree** at 3, and the **Peak Window Size** at 15 pts; Set the **Slope Threshold Peak Start** and **End** both at 0.0.
 - i. For **Yfiler_100rfu_3500**: set the peak amplitude thresholds at “100” for B, G, Y, and R dye filters and “400” for the O dye filter.
 - ii. For **Yfiler_200rfu_3500**: set the peak amplitude thresholds at “200” for B, G, Y, and R dye filters and “400” for the O dye filter.

NOTE: In some situations, such as reduced injection times, it may be necessary to analyze the O dye (internal size standard) at 100rfu if not all peaks are above 400. This change of analysis parameters shall be documented in the case file and the modified analysis method shall be exported and maintained in the electronic case record.

NOTE: If a one base difference between two alleles cannot be resolved (i.e. minor peak 1 base from major peak) after two injection attempts, then the Polynomial Degree and Peak Window Size can be adjusted to help resolve that specific sample. This change of analysis parameters shall be documented in the case file and the modified analysis method shall be exported and maintained in the electronic case record.

4. Select the **Peak Quality** tab. In the **Min/Max Peak Height (LPH/MPH)** box, enter “200.0” for both **Homozygous min peak height** and **Heterozygous min peak height**. Enter “40000” for **Max Peak Height (MPH)**. Enter “0.5” for Min peak height ratio in the Peak Height Ratio (PHR) box, “1.5” for Max peak width (basepairs) in the **Broad Peak (BD)** box, and “0.2” for the Cut-off Value in the **Allelic Ladder Spike** box. Under the **Allele Number (AN)** section, keep “2” for autosomal markers & Amelogenin and change to “1” for Y markers.

NOTE: Locus DYS385 is listed as an autosomal locus in the panels to differentiate that it can have up to two possible alleles in a single source Y sample. All other loci will be flagged as a mixture if more than one allele is present.

Do not alter the default settings in the SQ and GQ Settings tab.

GeneMapper® ID-X Parameters for the Y23® Analysis Method

1. In GeneMapper® ID-X, select GeneMapper® ID-X Manager.
2. Select the **Analysis Methods** tab, then click **New**.
3. **General** tab.
 - a. Enter a name for the method. Instructions provided below for methods named or similarly named: “Y23_100rfu_1.2” (used for unknown/questioned, negative control/reagent blank) and “Y23_200rfu_1.2” (used for known/reference, positive male control, and ladder).
 - b. From the drop-down list for Security Group, select **GeneMapper ID-X Security Group**.
 - c. Instrument type and a description may be entered.
4. **Allele** tab.
 - a. Select “PowerPlexY23_Bins_IDX_v1.2” from the Bin Set drop-down menu.
 - b. **Select “Use marker-specific stutter ratio and distance if available”.**
 - c. Enter the following:

Global Minus Stutter Distance	Tri	Tetra	Penta	Hexa
From	2.25	3.25	4.25	5.25
To	3.75	4.75	5.75	6.75
 - d. All other boxes, including Amelogenin Cutoff should have “0.0” entered.
5. **Peak Detector** tab.
 - a. In the **Ranges** box, for Analysis select **Full Range** from the drop-down menu. For Sizing select **Partial Sizes** from the drop-down menu; set the start size at 60 and the stop size at 500.
 - b. In the **Smoothing and Baseline** box, select **Light** for Smoothing and type in “51” for the Baseline Window.
 - c. For **Size Calling Method** select **Local Southern Method**.
 - d. In the **Peak Detection** box, set the **Min. Peak Half Width** at 2 pts, the **Polynomial Degree** at 3, and the **Peak Window Size** at 15 pts; Set the **Slope Threshold Peak Start** and **End** both at 0.0.
 - iii. For **Y23_100rfu_1.2**: set the peak amplitude thresholds for B, G, Y, R, and P at “100”, also set O at “100”.
 - iv. For **Y23_200rfu_1.2**: set the peak amplitude thresholds for B, G, Y, R, and P at “200”, set O at “100”.
 - e. Ensure the Use Normalization box is not checked.

NOTE: If a one base difference between two alleles cannot be resolved (i.e. minor peak 1 base from major peak) after two injection attempts, then the Polynomial Degree and Peak Window Size can be adjusted to help resolve that specific sample. This change of analysis parameters shall be documented in the case file and the modified analysis method shall be exported and maintained in the electronic case record.

6. Select the **Peak Quality** tab. In the **Min/Max Peak Height (LPH/MPH)** box, enter “100.0” for both **Homozygous min peak height** and **Heterozygous min peak height**. Enter “40000” for **Max Peak Height (MPH)**. Enter “0.5” for Min peak height ratio in the Peak Height Ratio (PHR) box, “1.5” for Max peak width (basepairs) in the **Broad Peak (BD)** box. Under the **Allele Number (AN)** section, keep “2” for autosomal markers and set “1” for Y markers. Set “0.2” for the Cut-off Value in the **Allelic Ladder Spike** box and enable Spike Detection for both Allelic Ladders and Samples.

NOTE: Locus DYS385 is listed as an autosomal locus in the panels to differentiate that it can have up to two possible alleles in a single source Y sample. All other loci will be flagged as a mixture if more than one allele is present.

7. Do not alter the default settings in the SQ and GQ Settings tab.
8. After completing these settings, click Save to save the changes and close the Analysis Method Editor.

Genemapper® ID-X Custom Control Import

As a quality control feature, GeneMapper® ID-X has the capacity to house up to 300 total analyzed lab reference samples, custom controls, and QC sample profiles in two separate databases (each with a 150 profile capacity).

IMPORTANT! Before adding custom control samples, review the samples manually and edit allele labels as needed to ensure that the profile is accurate. Profiles that include OL labels are not imported into the Profile Manager. Profiles that include numeric allele labels on peaks that are not true DNA peaks will affect concordance results.

IMPORTING THE REFERENCE PROJECT

1. Open GeneMapper® ID-X. Select the GeneMapper® ID-X **Manager** icon from the Project window toolbar (or from the Tools menu, select GeneMapper® ID-X Manager).
2. Select the **Projects** tab, then click **Import**.
3. Navigate to and select the appropriate project containing the profiles to be imported. Click **Import**. Ensure the appropriate Security Group is selected and click **OK**. Click **Done** to close the GeneMapper® ID-X Manager.

IMPORTING THE REFERENCE PROFILE(S)

1. In the Project window, click the **Open Project** icon. Select the imported project, then click **OK**.
2. From the Table Setting drop-down list, select **Import Reference Profiles**. The Sample table view changes to display only those columns required to add reference profiles to the GeneMapper® ID-X database.
3. In the Profile ID column of the Samples tab, click each cell and enter an appropriate Profile ID name (profiles are stored in the database under Profile ID not Sample Name).
4. Select the appropriate rows, then select **Tools, Add Profile**. Select the appropriate profile type: **Custom Control** or **Lab Reference**.
5. Click Close in the Add Profile Results dialog box to save the profile to the GeneMapper® ID-X database.

VIEWING PROFILES IN THE PROFILE MANAGER

1. In the Project window, select **Tools, Profile Manager**.
2. View the list of profiles in the Profile Manager window. Click '+' beside the Profile ID to view the genotypes stored in the GeneMapper® ID-X database.
3. Click **Close** to close the Profile Manager window and return to the Project window.

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STR FORMS

The worksheets approved for use in STR casework can be found on the WSP Portal.

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